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REACTIONS OF COLPODA DUODENARIA TO ENVIRONMENTAL FACTORS. II. FACTORS INFLUENCING THE FORMATION OF RESTING CYSTS[1]

(Four figures)

C. V. TAYLOR AND A. G. R. STRICKLAND
Stanford University

THE validity of results in biological experimentation obviously presupposes the reproducibility of the results under adequately standardized conditions. This would further imply that the conditions to be standardized should include the experimental material no less than the experimental methods employed.

Also, from recent studies (Taylor, Brown, and Strickland, 1936) relating factors of the environment to reactivities of *Colpoda duodenaria* which involve its protoplasmic reorganization, it is evident that the stage of this reorganization definitely enters into the requirement for adequate standardization of this experimental material. This is in line with the general assumption that the developmental stage of the cell or organism, as well as its genetic and environmental history, is an indispensable component of the conditions necessary for reproducible results.

And since protoplasmic reorganization occurs in the resting cysts of *C. duodenaria*, whose encystment and excystment we can induce at will, we are afforded the unique prospect of securing and maintaining, perhaps indefinitely, any desired quantity of experimental material of a known developmental stage whose genetic and environmental conditions may be satisfactorily standardized and so critically studied. Accordingly, were this prospect fully realized, we might then properly regard the induced resting cyst of this organism as a standard living cell, which should serve as an essential point of reference for experimental procedure and for reliable interpretation of experimental results. Our investigations, covering several years, on the nature and factors of induced cystment of *C. duodenaria* have been carried out with the foregoing purpose in view. Consequently, the results herein reported have been encouraging.

These results followed our efforts to determine some of the environmental factors which induce the change from a temporary cyst to a resting cyst and the conditions which must be controlled when uniform resting cysts are required. To this end we have investigated the effect (1) of the concentration of the *Colpoda* and (2) of the volume of the medium containing them, on the number of resting cysts induced.

[1] These studies were supported by a grant from the Rockefeller Foundation.

MATERIAL AND METHODS

For these experiments we have used the cultures of *C. duodenaria*, which have been maintained in thriving conditions in these laboratories for 7 years. Since we wished to determine the percentage of resting cysts formed in food-free medium by different concentrations of *Colpoda* in various volumes of medium, it was necessary to eliminate from the cultures, as far as possible, all organisms which would form cysts of diameter greater than 17 μ. Under the conditions of induced encystment, if the resulting cysts are larger than 17 μ, they cannot be relied upon to remain encysted. The larger cysts usually divide and excyst, producing two small daughters. Under certain conditions (unfinished studies), mainly of high concentration, cysts of greater than 17 μ diameter can be induced to remain encysted, i.e., to form resting cysts; but for these experiments such sizes were, as far as possible, eliminated.

Colpoda of the required size were produced by controlling the supply of food, the aim being to cover the bottom of the dish with large numbers of quadrigenic division cysts of diameters about 28 or 30 μ. The daughters are then of the right size for these experiments.

Colpoda in sufficiently food-free medium were obtained by two methods, both of them quite satisfactory. When small numbers were required, the ciliates were transferred with sterile micropipettes through three or more dishes of sterile medium. When greater numbers were needed, the culture was washed three or more times in the centrifuge. It is claimed not that these organisms were sterile but that they were so free from bacteria that in a nonnutritive medium, such as was used in this work, the effect of food was negligible.

In the first series the volume of medium, called, for convenience, the "drop," was $\frac{1}{4}$ cc., and the concentrations of *Colpoda* ranged from zero to 4,000 per cubic centimeter. For concentrations up to 500 per cubic centimeter the required number of protozoa was picked up in a pipette calibrated to $\frac{1}{4}$ cc. For higher concentrations the washed culture was diluted approximately to the concentration required, and a drop of $\frac{1}{4}$ cc. was picked up in a pipette and transferred to a Columbia dish for observation. Since food was absent, the *Colpoda* all encysted; when encystment was complete, the cysts were counted. In the few cases in which the number of cysts was too great to be counted accurately, separate cultures of cysts were counted with care, and thereafter the total number was estimated.

Soon after encystment was complete, excystment, if any, began. Unpublished studies show that, in the absence of food, these excysted organisms re-encyst and that some of them may form resting cysts. This re-encystment takes place in a different environment from the first. We are concerned in this paper only with the phenomena of the first encystment, and so at intervals the excysted *Colpoda* were removed with an extremely small pipette. Twenty-four hours after encystment the number of resting cysts was counted. This number, added to the number of ciliates removed, gives a useful check on the total of organisms originally in the drop.

The same experiment was repeated with drops of 1/40 cc. In this case it was found advisable to put the drops on the cover of a small Petri dish. Evaporation was hindered by additional moisture in the dish.

The same experiment was repeated with drops of 1/400 cc. In this case each drop was placed on a cover slip, which was reversed over a deep hollow-ground slide containing

sufficient moisture to saturate the atmosphere. The cover slip was sealed onto the slide with water, which was replenished when necessary. Encystment was usually complete after 3 hours; and excystment, if any, after 6 hours. The cover slips were then immersed on a rack in a large volume of sterile balanced salt solution. The following morning only the resting cysts remained on the cover slips.

The same technique was employed for drops of 1/4,000 cc., except that usually 4 drops were put on each cover slip. When more than 1 drop was put on a cover slip, the outline of each drop was marked with India ink. Drops of 1/40,000 cc. were much more difficult to prepare. However, with practice and a suitable point on a micropipette calibrated to 1/4,000 cc., it was possible to put onto a cover slip one-tenth of 1/4,000 cc. in 10 drops of approximately equal size. Drops obviously too large or too small were discarded.

All experiments were carried out in a constant-temperature room at 20° C. The relative humidity was normally maintained at 65 per cent; but in order to reduce evaporation, it was raised to 80 per cent and more while the small drops were being prepared.

To study the formation of resting cysts in drops much smaller than 1/40,000 cc., a micromanipulator with injection apparatus (Taylor, 1925) was used. To it was attached a capillary tube of soft glass, into which was drawn a drop of food-free medium containing one or more *Colpoda*. On each side of the drop, but separated from it by a small air space, was another drop of medium to prevent evaporation. The piece of the capillary containing the drops was then broken off, and the two ends were plugged with soft paraffin. The small section of capillary was then affixed to a cover slip with wax; the cover slip was reversed onto a hollow-ground slide for convenience in handling.

EXPERIMENTAL

By the methods just described, 67 drops of $\frac{1}{4}$ cc. each were tested to determine the percentage of resting cysts formed at various protozoan concentrations. When this percentage of resting cysts is plotted on linear paper against the concentration of ciliates, the curve is apparently logarithmic. Figure 1 shows the curve for 1/4,000 cc. The curves for $\frac{1}{4}$ cc. and all other volumes are similar. This is confirmed by plotting the percentage of resting cysts against the concentration on a logarithmic scale. For this purpose the 67 cases in which the drop was $\frac{1}{4}$ cc. were divided into five groups. In each group the percentage was determined from the mean number of resting cysts formed and the mean concentration of protozoa. The curve (Fig. 2) is then a straight line, which cuts the axis of the abscissas at about 16 organisms per cubic centimeter and the 100 per cent line at about 6,500 per cubic centimeter. From this it appears that, when 1,625 *C. duodenaria*, i.e., 6,500 per cubic centimeter, in sizes too small to result in binary fission after encystment, are present in $\frac{1}{4}$ cc. of food-free balanced salt medium, they will all form resting cysts. When 4 such (or less) are present in the same amount of medium, no resting cysts will be formed, but the cysts induced by the absence of food will all reorganize and excyst.

To determine the curve for drops of 1/40 cc. we have 138 cases, divided into seven groups. When the mean percentage of resting cysts is plotted against the logarithm of the mean concentration in each group on a logarithmic scale, we have seven points, which appear to lie on a straight line parallel to the $\frac{1}{4}$ cc. line (see Fig. 2). We see that no resting cysts are formed unless the concentration is 225 or more per cubic centimeter,

i.e., 5.6 organisms in the drop, and that it requires a concentration of 105,000 per cubic centimeter, i.e., 2,625 per drop, to induce permanent encystment in all of them.

For drops of 1/400 cc. we have 279 cases, divided into nine groups. The concentration must be 850 per cubic centimeter, i.e., 2.1 per drop, before any resting cyst is formed, and 410,000 per cubic centimeter to induce 100 per cent permanent encystment.

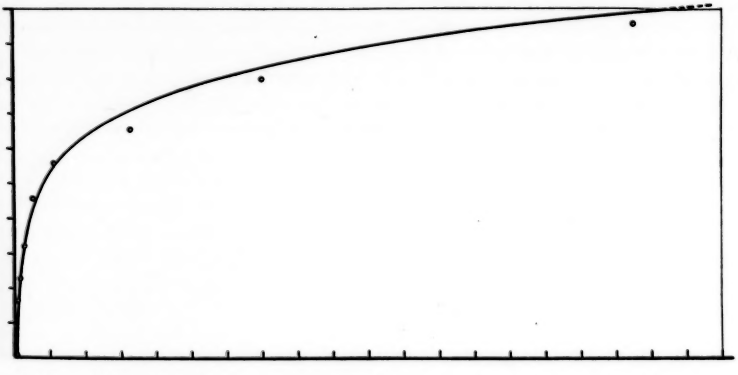

FIG. 1.—Induced resting cysts: percentage of concentration in 1/4,000 cc. of medium

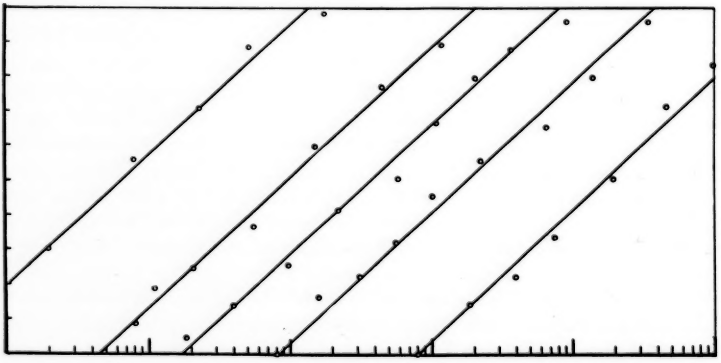

FIG. 2.—Resting cysts: percentage plotted against log concentration of protozoa in various volumes of medium.

The curve is also a straight line parallel to the former two. Similarly, for 1/4,000 cc. we have 466 cases in nine groups. One organism per drop, i.e., 4,000 per cubic centimeter (34 cases), never forms a resting cyst, and a concentration of 1,950,000 per cubic centimeter is necessary for 100 per cent permanent encystment. For drops of 1/40,000 cc. we have 169 cases in seven groups. No resting cysts are formed at a concentration of 40,000 per cubic centimeter, and for 100 per cent permanent encystment a concentration of 17,200,000 per cubic centimeter is necessary.

The curves for all these drops of different volumes appear to be parallel straight lines on semilogarithmic paper, thus showing that the smaller the drop the greater the concentration of protozoa necessary to induce formation of resting cysts.

It will be sufficient to give (Table 1) the actual figures and calculations for any one of these curves, e.g., for 1/4,000 cc., as the others are similar, and the points can be seen in Figure 2.

TABLE 1

(Size of drops, 1/4,000 cc.)

Concentration of *Colpoda* per Cubic Centimeter	Number of Cases	Mean Concentration	Mean Percentage of Resting Cysts	Standard Deviation σ	Standard Error*
4,000	34	4,000	0	0	0
8,000	104	8,000	16.4	22.45	2.21
12,000–20,000	107	15,800	22.5	27.8	2.07
24,000–36,000	41	28,400	32.0	27.3	4.26
40,000–60,000	38	57,100	45.5	22.75	3.68
64,000–236,000	71	112,800	55.5	19.5	2.32
240,000–396,000	19	331,000	65.2	14.6	3.34
400,000–1,196,000	38	700,000	79.5	16.45	2.66
1,200,000 and over	14	1,753,000	95.7	5.47	1.46

* The mean standard error of the mean is ±3.32 for ½ cc., 3.59 for 1/40 cc., 3.07 for 1/400 cc., 2.50 for 1/4,000 cc., and 3.72 for 1/40,000 cc.

In Figure 3 we have plotted the minimum protozoan concentration (taken from Fig. 2) necessary to induce 100 per cent formation of resting cysts in each size of drop. The curve resembles a hyperbola whose asymptotes are nearly the co-ordinate axes. This shows clearly that, as the volume of medium decreases, the concentration of organisms necessary to induce 100 per cent formation of resting cysts increases very rapidly. We have omitted from Figure 3 the volumes ¼ cc. and 1/40,000 cc. because, to include them, it would have been necessary to reduce the units of both axes tenfold.

The question now arises: Can one *C. duodenaria* so condition its own environment that it will itself form a resting cyst? From Figure 3 it appears that this might occur if the drop were sufficiently small. If the drop is extremely small, the concentration required to induce 100 per cent formation of resting cysts should be correspondingly enormous. When a minute drop containing one or more *Colpoda* is isolated in a capillary tube, it is possible to obtain very high concentration in very small volumes of medium. We have 100 cases for consideration, in which the drops ranged from 5×10^{-7} cc. upward. In no case did one *Colpoda* in a drop form a resting cyst. As the number of protozoa increased up to 40, the percentage of resting cysts increased approximately along a logarithmic curve. It was not possible, owing to technical difficulties, to increase the number of ciliates or to diminish the volume of medium sufficiently to obtain 100 per cent formation of resting cysts; or at least the time required to do so seemed incommensurate with the value of the expected results.

Figure 3 also suggests that one *Colpoda* in a drop might induce itself to form a resting cyst if the volume of medium were sufficiently large. This is supported by Figure 4, which illustrates the actual number of organisms necessary to induce 100 per cent of resting cysts in drops of different sizes.

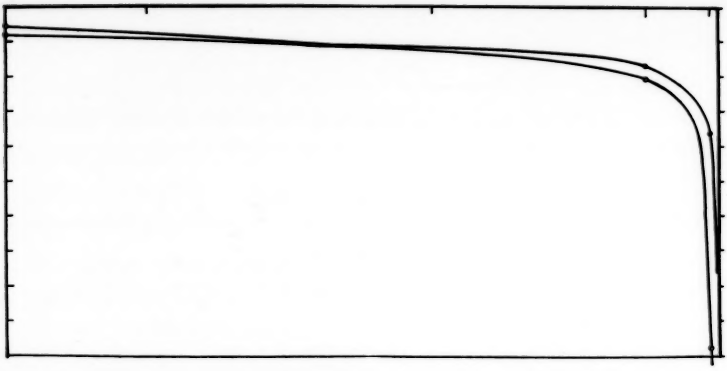

FIG. 3.—Graphs indicating possible relationship between induction of resting cysts and the surface: volume ratio of the drops. (*a*) Minimum concentration to induce 100 per cent of resting cysts plotted against volume of the drop (○); (*b*) surface-volume ratio (air-water surface) plotted against volume of the drop (●).

Vol.	Surface-Volume Ratio	Concentration
1/4	7.24	6,500
1/40	15.5	105,000
1/400	33.6	410,000
1/4,000	72.4	1,950,000
1/40,000	155.0	14,200,000

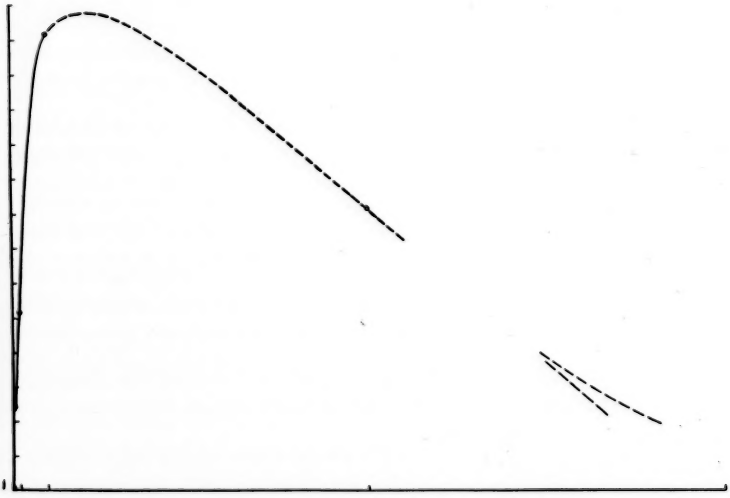

FIG. 4.—Number of *Colpoda* per drop necessary to induce 100 per cent of resting cysts in drops of various volumes.

We have not sufficient data to extrapolate this curve beyond $\frac{1}{4}$ cc.; but in order to find out if it cuts the axis of the abscissas in the neighborhood of 1 cc., we made thirteen tests of one *Colpoda* in $\frac{1}{2}$ cc. and eight tests of one organism in 1 cc. In no case was a resting cyst formed. Since it is very difficult to find a small cyst in such large volumes of medium, these tests were discontinued for the present.

In Figure 3 we have also plotted the surface-volume ratio (i.e., the air-water surface) for the different volumes of medium, assuming, therefore, that the drop is a segment of a sphere and that the height of the segment is one-third of the radius of the sphere. The scale of ordinates for this curve is on the right side of the figure. The striking similarity between the two curves in Figure 3 will be discussed later.

DISCUSSION

As previously shown (Taylor and Strickland, 1938), when food is removed from their medium, all *C. duodenaria* will encyst. If large enough, they will ordinarily then undergo fission and emerge from the cyst as two, four, or even eight daughters, according to size of the cyst. Smaller *Colpoda*, forming cysts of diameter less than about 17 μ, do not divide; but, depending upon conditions described in foregoing paragraphs, they may either (1) redifferentiate in due course and emerge as normal free-swimming forms or (2) form resting cysts which are readily identifiable, owing to changes in their appearance and to a perceptible diminution in size. The latter will remain dormant until redifferentiation is initiated by a suitable stimulus.

The experimental data herein presented have had to do primarily with resting cysts. For purposes of comparison throughout this discussion, however, it will be well to bear in mind also the other type (see [1] above) of nondivision cysts, which are only temporary. Following emergence, if no food is available, the free-swimming forms again encyst. This cycle is repeated again and again at fairly regular intervals, with a diminishing size of the cyst.

Our experimental results recorded above indicate that there may be no intrinsic difference between those *C. duodenaria* that will form these temporary cysts, on the one hand, and those that will form resting cysts, on the other. Which of these two types of cysts will be formed may depend rather on the conditioning of the food-free medium at the time encystment takes place, for in a given volume of this medium the percentage of resting cysts formed depends on the number of organisms present, i.e., the concentration of *C. duodenaria* per cubic centimeter of the food-free medium.

This significant finding is well demonstrated in the curve of Figure 1, as plotted on linear paper for various concentrations of *Colpoda* in drops of 1/4,000 cc. of balanced salt medium. In all concentrations below 4,400 per cubic centimeter redifferentiation and emergence ensue, while in all higher concentrations up to 1,950,000 per cubic centimeter the percentage of resting cysts formed increases with increasing concentration, at first rapidly and then more and more slowly, up to 100 per cent of resting cysts. In concentrations above 1,950,000 per cubic centimeter there are, of course, 100 per cent of resting cysts; but the extrapolated continuation of the curve may indicate that such concentrations produce a surplus of some unknown factor or factors, which theoretically, could induce the formation of more resting cysts than there are organisms in the drop.

Certain technical difficulties have thus far not been fully overcome in the transfer of the organisms to a balanced salt, food-free medium. For each test the culture of *Colpoda* has to be washed as free as possible of bacteria and of organic solutes (Taylor and

Strickland, 1938) by repeated use of the centrifuge. Moreover, there are commonly present a few *Colpoda* large enough to form digenic division cysts, whose emerged progeny thus count doubly as free-swimming forms and reduce the percentage of resting cysts.

To these and other technical difficulties may be ascribed the relatively high values of the standard deviation of the mean (Table 1). This is obvious especially in tests where a small change in the concentration produces a large change in the percentage of resting cysts (cf. steep portion of curve in Fig. 1) or where the few organisms in a small drop (1/4,000 cc.) represent in the calculation a large number per cubic centimeter. For example, two organisms in such a drop correspond to a concentration of 8,000 organisms per cubic centimeter; and since the possible results are 0 per cent, 50 per cent, or 100 per cent of resting cysts, a large standard deviation can be expected. The maximum value of σ for these tests is ± 27.8 per cent; but as the number of organisms in the drop increases, σ falls progressively to ± 5.47 per cent. Owing to the large number of cases in each group of tests, the standard errors of the mean are reasonably small.

It is evident from Figure 1 that, for a given volume of food-free medium, the percentage of resting cysts formed is some function of the concentration of the organisms. In Figure 2, however, the graphs show that in drops whose sizes vary by a factor of one-tenth, from $\frac{1}{4}$ cc. to 1/40,000 cc., the ratio values of the percentage of resting cysts to concentration of organisms vary with the size of the drops. On semilogarithmic paper the graphs are all straight lines and parallel. These data indicate that the surface of the drop, as well as its volume, is a limiting factor, since the smaller the drop the greater is the concentration of organisms required to induce the formation of a given percentage of resting cysts.

Further demonstration of this relation of the size of the drop of food-free medium to the concentration of organisms in the induced formation of resting cysts of *C. duodenaria* is given in the curves of Figure 3. In the first of these, marked *a*, the concentration of *Colpoda* (in millions per cubic centimeter) necessary for the induction of 100 per cent resting cysts is plotted on linear paper against the volume of the drops. The resulting hyperbolic curve is strikingly similar to the second curve, marked *b*, of the surface-volume ratio plotted also against the volume of the drops. Curve *a* on logarithmic paper gives approximately a straight line, so that it is nearly a rectangular hyperbola. The curves *a* and *b* are, however, so nearly alike that it seems a plausible hypothesis that the surface-volume ratio is indirectly a controlling factor in the induction of resting cysts. If the surface-volume ratio is plotted on logarithmic paper against the corresponding concentration of *Colpoda* necessary to induce 100 per cent of resting cysts, the five points obtained are also approximately on a straight line.

Extrapolation of the arms of curve *a* in Figure 3 might suggest the possibility of preparing, on the one hand, a drop small enough (cf. vertical arm) or, on the other hand, a drop just large enough (cf. horizontal arm) so that in either case the limiting factor might enable one *Colpoda* so to condition the drop that the organism would be induced to form in it a resting cyst. Since the volume of the cyst would be about 2,500 μ^3, or 2.5×10^{-9} cc., and since for inducing resting cysts the required concentration of *Colpoda* increases nearly tenfold when the volume of the drop is decreased from 1/4,000 cc. to 1/40,000 cc., then by extrapolation it would appear that the volume of the theoretical drop in which one *Colpoda* would induce itself to form a resting cyst might be smaller than the organism itself.

An extrapolation of the horizontal arm of curve *a* in Figure 3 is indicated by the dotted line in Figure 4. Assuming that this arm very closely approximates the axis somewhere between $\frac{1}{4}$ cc. and 1 cc. and that, accordingly, one *Colpoda* might induce itself to form a resting cyst in a drop of $\frac{1}{2}$ cc., or in a drop of 1 cc., thirteen tests were carried out with one organism in $\frac{1}{2}$ cc., and eight tests with one organism in 1 cc. In all tests the results were apparently negative, although the difficulties of following the fate of an organism so small in such large drops are obvious.

It is, of course, quite possible that in either or both of these extreme parts of curve *a* in Figure 3, the surface-volume factor for induced resting cysts may be modified by some other unknown factor or factors so that the curve might thereafter tend to become parallel to the co-ordinate axes. In such cases one could then expect that a minimum constant number of *Colpoda* would be necessary to produce 100 per cent of resting cysts and that this number might in either case be greater than one.

The experimental results reported in this paper and analyzed by graphs in the several figures appear, therefore, to establish a relation between the surface-volume ratio of the food-free drops containing *C. duodenaria* and the induced formation of its resting cysts. Just what specifically is the essential factor or factors involved in this relation between the environmental medium and the reacting organism is at present not clear.

Several possibilities are fairly obvious. Since to induce resting cysts the concentration of organisms must be increased as the size of the drop is decreased, i.e., as the surface-volume ratio of the drop increases, one might at once suppose that some essential volatile substance, such as CO_2, is escaping into the gas phase, or that the O_2 diffusing from the gas phase into the drop may counteract the environmental factor(s) that induce resting cyst formation. Thereby, in either case, the food-free medium becomes modified at a rate varying approximately with the surface-volume ratio of the drop. And since this modification of the medium would presumably tend to inhibit the formation of resting cysts, then, in order to offset this modification, the concentration of *Colpoda* would have to be increased as the surface-volume ratio of the drops is increased, i.e., as the drops are made smaller and smaller.

This could, of course, presuppose that the organisms are adding some "*r*-substance" or substances to the medium which, at threshold concentrations, condition the medium and, in turn, induce the formation of resting cysts. Carbon dioxide or some unoxidized metabolite(s) might be the *r*-substance(s) in question. In any case, its concentration would evidently be affected not only by the rate of its production (hence by the relative number of organisms present) but also, for small drops, by its rate of escape through the surface of the drop, or, if surface active, by its accumulation at the drop's surface, or by its change through oxidation or otherwise to a biologically inactive state. For the large drops, however, as represented by the horizontal arm of curve *a* in Figure 3, the threshold concentration of this hypothetical substance, at a given rate of its production, might depend chiefly on its diffusion, since the surface-volume ratio should here become less and less a limiting factor.

These considerations obviously relate to the possible origin, nature, and identity of the factor or factors that activate *Colpoda* to form resting cysts. Investigations now under way may provide these essential data on the activating agent(s) thus operative. One may well bear in mind, however, that it is the reacting protoplasm, rather than activating agents, which primarily concerns the biologist. Clearly enough, for example, *Colpoda* may form a resting cyst instead of a temporary cyst, previously referred to,

upon reacting to its conditioned medium, so that the former cyst must differ from the latter, owing to some change in its protoplasmic state. This state may then, at will, be reversed upon the addition of a suitable excystment medium.[2]

Some things are known about the cystment process in *Colpoda* to which brief reference may here be made as a possible means of better understanding this essential difference between its resting cyst and its temporary cyst. A detailed study has been made (Taylor and Garnjobst, 1939) of protoplasmic reorganization in *C. duodenaria* during fission. Here all protoplasmic organelles, such as cilia, and the fibrillar system, are resorbed, and new organelles are redifferentiated in the resulting daughters. No detailed study of structural changes during its formation of resting cysts has, as yet, been made; but results thus far obtained make it appear altogether probable that reorganization then also occurs, as during fission. Assuming this to be so, its possible significance will be referred to farther on.

From studies on the effects of X-rays on normal excystment time of *Colpoda* (Taylor, Brown, and Strickland, 1936) it was found that for a given dose of 38,400 *r*-units applied at various times during the first hour of experimentally induced excystment, the excystment time was increased in each case approximately threefold. But the same dose applied at intervals after the first hour was less and less effective, so that the excystment time for organisms X-rayed near their emergence was approximately normal. Evidently, then, the X-ray effect varied with the stage of excystment process when the dose was applied.

It was further shown (Brown and Taylor, 1938) that during the first 20–25 minutes of induced excystment, the excystment time was dependent upon the concentration of the excystment medium but independent of the O_2 tension. For the remainder of the first hour, however, the excystment time varied with the O_2 tension but was independent of the concentration of the excystment medium. This would indicate that at the beginning of induced excystment some essential ingredient of the excystment medium was diffusing into the protoplasm with which it combined, thus to initiate the excystment processes requiring oxygen. If, as we assumed, this combination of diffused ingredient and protoplasm involved enzyme synthesis, then we could further suppose that the essential ingredient added from the excystment medium was conceivably a co-enzyme (prosthetic group), which combined with a protein constituent of the protoplasm, thereby forming the required enzyme for the initiation of processes leading to visible protoplasmic redifferentiation and excystment.

Could these events initiating *re*differentiation and excystment be thus reasonably postulated, then it would seem to follow that the formation of a resting cyst may involve in its *de*differentiation an inactivation of certain enzyme(s) whose degradation was brought about by a threshold concentration of the hypothetical *r*-substance(s) in the conditioned medium. This might imply, let us suppose, a splitting-off and loss, from the protein component of such enzymes, of the complementary and simpler prosthetic component which, before excystment will occur, must ordinarily be contributed to the resting cyst from a suitable excystment medium. Temporary cysts, on the other hand, may not, in the absence of a sufficiently conditioned medium, suffer this degradation of the enzymes that activate redifferentiation and excystment, so that excystment in these temporary cysts ensues spontaneously.

[2] Haagen-Smit and Thimann recently found (1938) that the potassium or sodium salts of several organic acids were effective agents for excystment of *C. cucullus*.

This hypothesis would seem to agree thus far very well with the experimental data herein presented and previously reported on induced cystment in *C. duodenaria*, and it should provide a satisfactory working basis for our further studies along this line which are now in progress.

Finally, brief mention might be made of the bearing our experimental results may have on those published by several others who have studied cystment in this and other ciliates:

Penn (1934) agrees that crowding induces encystment (in *Pleurotricha lanceolata*) but adds that the effect is due to accumulation of waste products. We have shown (Taylor and Strickland, 1938, Expt. II) that, for *C. duodenaria*, at least, crowding does not induce encystment when sufficient food is present in the medium, and we have further shown (*ibid.*, Expt. V) that a 1 per cent solution of sterile yeast extract can provide sufficient food to maintain the free-swimming state for a comparatively long time. If these observations hold good for *Pleurotricha*, then the three cases in Penn's Table II are open to a different interpretation, namely, (*a*) 1 animal in 1 ml. of sterile rye extract gave only 1.03 per cent of cysts, as, indeed, would be expected if the rye extract were supplying some nourishment; (*b*) 10 animals in 1 ml. of rye extract gave 39.33 per cent cysts, as we might expect, since 10 animals would use up the available food ten times as quickly as 1 animal; (*c*) 1 animal in 1 ml. of rye extract plus excretion products could also be expected to give a high percentage of encystment, because the 10 *Pleurotricha* left for 24 hours in the extract to accumulate excretion products would have used up much of the available food.

Barker and Taylor (1931) state that crowding is of primary importance for the encystment of *Colpoda;* our data confirm this statement, with the reservation that the medium must be free of food. If, as we believe, the protozoa used in their work were the progenitors of the one we are now using under the name of *C. duodenaria* (Taylor and Furgason, 1938), then, in the light of more recent investigations, Table V in their paper seems to offer insufficient data on which to base the foregoing conclusion. In the first 5 cases (abundant bacteria) only a varying number of division cysts, but no permanent cysts, should have been formed. In the next 3 cases (no bacteria) the first has such a low concentration that the protozoa were probably undergoing the rhythmic encystment and excystment (Taylor and Strickland, 1938, p. 401), ending in death from depletion about the fifth day; the second has sufficient concentration, if in food-free medium, to give 50 per cent of permanent cysts in about 5 hours, so probably there was some food available; the third (3,600 per cubic centimeter) in food-free medium should make about 90 per cent permanent cysts within 4 or 5 hours.

Although our data offer no definite evidence of any inhibiting factor other than the presence of food, it is interesting to note that Beers (1927) found for *Didinium nasutum* an inhibition of encystment when the excretion products of *Paramecium* were present in the medium. It is, however, not clear whether there may not have been in the medium sufficient nutriment, such as organic solutes, living or dead bacteria, and other particles, to maintain the free-swimming state and prevent encystment for some time. In Part I of this series we have shown that the metabolites of *Colpoda* and *Pseudomonas* accumulating for 16 weeks do not seem to have any effect on encystment.

Mast and Ibara (1923) state: "Rapid and extensive reproduction of *Didinia* confined to a small space seems to favor encystment and it may be that this is owing to accumulation of waste products." Unless there is at all times sufficient food in the medium

to maintain the culture, we may suppose that crowding of *Didinium* when the food is used up induces encystment, as in *Colpoda*. Their tentative explanation would then be open to question.

SUMMARY

This study of the conditions which induce the formation of resting cysts of *C. duodenaria* reveals that:

1. The percentage of resting cysts formed in a given volume of food-free medium is proportional to the logarithm of the protozoan concentration.

2. The smaller the volume of medium, the greater must be the concentration of protozoa to induce all of them to form resting cysts.

3. For drops of medium from $\frac{1}{4}$ cc. down to 1/40,000 cc. the curve illustrating the variation of this concentration with the volume of the medium resembles a hyperbola.

4. There is a striking resemblance between the curve of concentrations and a curve giving the surface-volume ratio for the various volumes of medium.

5. It is theoretically possible for one *Colpoda* to induce itself to form a resting cyst in extremely small or in large volumes of food-free medium.

6. The possibility of an encystment-promoting factor is briefly discussed.

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CHEMICAL MEDIATION IN CRUSTACEANS. II. THE ACTION OF ACETYLCHOLINE AND ADRENALIN ON THE ISOLATED HEART OF PANULIRUS ARGUS

(Five figures)

JOHN H. WELSH
Bermuda Biological Station for Research, Inc., and
Biological Laboratories, Harvard University

THE presence of relatively large amounts of acetylcholine (Welsh, 1938 and 1939) and choline esterase (Bacq and Nachmansohn, 1937; Marnay and Nachmansohn, 1937) in nervous tissues of decapod crustaceans suggests that chemical mediation of nervous impulses by acetylcholine may occur in this group of invertebrates as it does in the vertebrates. Attempts, thus far, to demonstrate an effect of acetylcholine at the neuromuscular junction of skeletal muscle of crustaceans have not been conclusive (e.g., Katz, 1936), but it has been shown to act on the isolated hearts of *Maia* and *Carcinus* in concentrations of physiological significance (Welsh, 1939). The action on the crab's heart is the opposite of that on the vertebrate heart, since acetylcholine excites the former and inhibits the latter type of heart. Also of interest are the opposed actions of acetylcholine and adrenalin on the vertebrate heart and the excitatory action of both of these substances on the crab's heart.

MacLean and Beznák (1933) had earlier studied the action of acetylcholine and adrenalin on the heart of the crayfish and had extracted a substance having the properties of a choline ester from this heart; but this work was unknown to the writer when the work on *Maia* and *Carcinus* was being done, and not until the present study had been completed did the paper come to my attention. Without knowledge of the work of MacLean and Beznák it seemed desirable to determine the action of acetylcholine and adrenalin on the heart of a representative of the Macrura and especially to obtain more exact quantitative data with a view to extending our knowledge of the mode of action of these two common mediator substances. Certain difficulties were encountered in dealing with the heart chosen for this study; and it was found impossible, in the limited time, to obtain all the desired data on the separate and combined actions of acetylcholine and adrenalin. It was possible, however, to demonstrate that the actions of these two substances, although excitatory, were not identical, and to obtain some idea of the effects of various concentrations. An idea of the arrangement and action of the local nervous system of the decapod heart was also obtained, this being necessary in any detailed analysis of the control of beat of this type of heart.

Certain of the more important findings form the basis of the present paper. A grant from the Milton Fund of Harvard University made this work possible.

MATERIALS AND METHODS

The heart of *Panulirus argus* (Latreille), the sea crawfish, or spiny lobster, of the region of the West Indies, was chosen for the present study. The large size of this heart was not found to be as much of an advantage in the perfusion studies as had been expected,

but it was ideal for determining the arrangement of the ganglion cells in the heart of a marine decapod.

To prepare the heart for perfusion, it was first exposed by removal of a portion of the carapace dorsal to the heart. Preliminary to this, the legs had been cut off and the animal allowed to bleed. Flushing out the pericardial space with perfusion fluid prevented the coagulation of any remaining blood. An incision in the dorsal abdominal artery permitted the insertion of a cannula directly into the cavity of the heart. The ligature, which secured this cannula, was so placed that it also included the sternal artery. This prevented the rapid escape of fluids by way of this blood vessel and helped in maintaining a slight pressure within the heart, which is quite necessary for its normal functioning when isolated. A second thread tied off the ophthalmic and antennary blood vessels and was used to attach the heart to the writing-lever. The ligaments of the heart were then cut, and it was placed in a perfusion bath and the cannula connected, by a two-way stopcock, to a large perfusion bottle and a perfusion tube. By means of the latter, small amounts of test fluids could be introduced to the heart. The rates of flow from these two were adjusted by keeping them at appropriate levels above the heart and by adjustable clamps placed on the rubber connectives leading to the stopcock. An illustration of such a setup may be seen in the first paper of this series (Welsh, 1939).

Since an analysis of the ionic composition of the blood of *Panulirus* was lacking, a perfusion fluid having the composition of that used for *Carcinus*, the green crab, was tried. This contained 100 parts of 0.6 M NaCl, 2.5 parts of 0.6 M KCl, 4.0 parts of 0.4 M $CaCl_2 \cdot 6H_2O$, 7.0 parts of 0.4 M $MgCl_2$, and was brought to pH 7.0 with $NaHCO_3$. On the first few hearts this seemed to be adequate, as it maintained a regular beat for periods of 10 or more hours. During later work, however, it was obvious that it was not an entirely appropriate mixture. Changes in the potassium and magnesium were tried, and a reduction to 2.5 parts of 0.4 M $MgCl_2$ helped most in preserving a regular beat over a period of time. Future work on the heart of *Panulirus* should be preceded by an analysis of its blood and the development of a more satisfactory perfusion medium. Stock solutions of atropine sulphate, eserine sulphate, acetylcholine chloride, and adrenalin chloride were made at frequent intervals, usually daily; and appropriate dilutions with the perfusion medium were made just before using. The stock solution of acetylcholine was kept at a pH of 4.0.

The arrangement of ganglion cells and fibers in the heart was demonstrated by staining with methylene blue. The heart was split lengthwise along the ventral side, pinned out flat, and covered with perfusion fluid made slightly acid with HCl (pH 4.0–5.0). To this was added 0.5 per cent methylene blue in the proportion of 15–20 drops of methylene blue to 100 cc. of perfusion fluid. The preparation was placed in a refrigerator for 8–12 hours and then examined. Excellent results were obtained, and it is believed that this was partly due to keeping the preparation at a low temperature.

THE ACTION OF ESERINE, ATROPINE, AND ACETYLCHOLINE

If the local nervous system of the heart of *Panulirus* is cholinergic, as seems to be the case in *Maia*, *Carcinus*, and the crayfish, it should be possible to demonstrate this by the appropriate use of eserine, atropine, and acetylcholine. Eserine is known to potentiate the action of acetylcholine by preventing its hydrolysis by choline esterase. Atropine abolishes the action of acetylcholine when it has a so-called "muscarin effect"; and the

evidence from *Carcinus* indicated that this, rather than the "nicotine effect," characterizes the action of acetylcholine in the decapod heart.

When the isolated heart of *Panulirus* was perfused with eserine 10^{-5}, two effects were observed, depending on the previous state of the heart. If the heart had been beating in a somewhat irregular manner (e.g., exhibiting a series of grouped beats), a regular beat

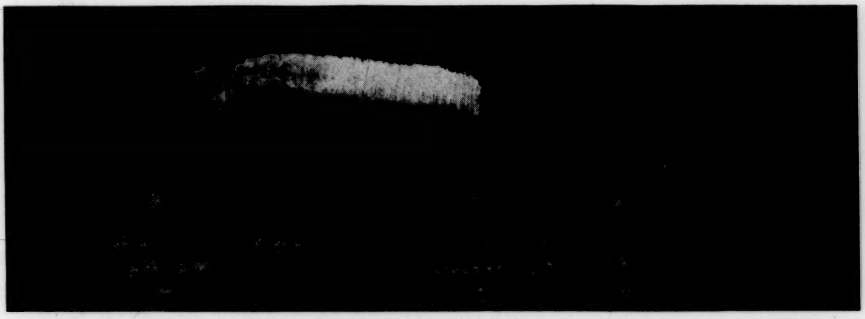

FIG. 1.—Record of the effect of perfusion with acetylcholine 10^{-6} between first and second arrows, and then with atropine 10^{-5}, followed by normal perfusion medium. Time interval = 5 seconds in this and subsequent records.

FIG. 2.—Showing the effect of acetylcholine 10^{-5} on the same heart from which record in Figure 1 was obtained.

would be completely restored. If the heart had been beating in a regular manner, an observable increase in frequency occurred. After a period of perfusion with eserinized perfusion fluid, followed by a period of washing, a second trial with eserine had little effect on the heart.

Acetylcholine 10^{-10} to 10^{-9}, which were the lowest concentrations used, had effects similar to those produced by eserine, namely, the restoration of a normal beat or a slight

increase in frequency. Acetylcholine 10^{-8}, after eserinization, caused an increase in frequency of beat and a slight increase in tonus[1] of certain hearts, while acetylcholine 10^{-7} caused a marked increase in frequency and a decrease in amplitude. Further increases in the concentration of acetylcholine produced further changes, ending in complete systolic stoppage with a concentration of 10^{-4}, from which the heart would not recover until washed for several minutes. A series of records of the effects of high concentrations on the same heart are shown in Figures 1–3. Obviously, such effects as these produced by high concentrations of acetylcholine are not "normal," but they are chosen to illustrate the essential differences in the action of acetylcholine and adrenalin on this heart, which was one of the purposes of this work.

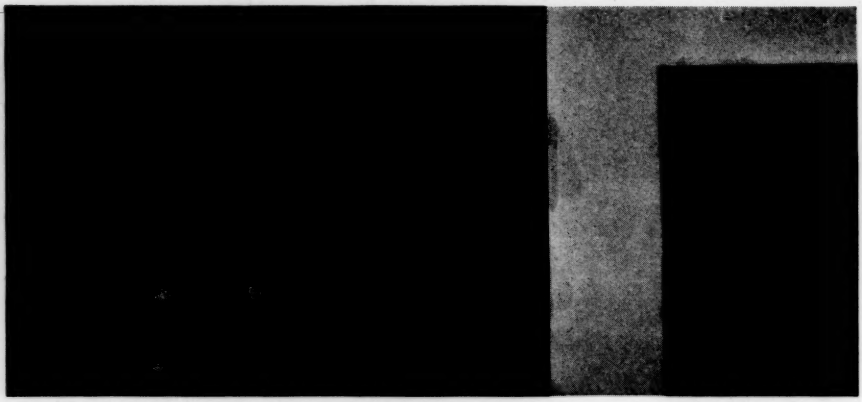

FIG. 3.—Showing the effect of acetylcholine 10^{-4} on same heart as Figures 1 and 2. A period of 6 minutes separates the two portions of the record.

Atropine reduces the rate of beat or stops the heart of *Panulirus*, the effect depending on the concentration. In Figure 1 may be seen a record of the effect of atropine 10^{-5} following a period of perfusion with acetylcholine. If the heart is perfused with atropine 10^{-5} for a few minutes and then perfused with acetylcholine, the stimulating action of the acetylcholine is largely abolished, as in *Carcinus*.

THE ACTION OF ADRENALIN

Adrenalin had been found by Bain (1929) to accelerate the heart of *Carcinus* in a concentration of 1:10,000,000. No attempt was made to find the limit of sensitivity of the heart of *Panulirus* to adrenalin, but certain hearts showed a striking response to adrenalin 10^{-6} to 10^{-5}. Figure 4 is a record showing the effect of perfusing a heart for 2 minutes with adrenalin 10^{-5}. Marked acceleration was followed by complete diastolic stoppage during 4 minutes of washing; then a beat of greatly increased amplitude and frequency appeared. Although the frequency rapidly returned to normal, the increased amplitude was noticeable 20 minutes after perfusion with the adrenalin had ceased.

[1] Whether a slight rise in base line is the result of a tonus change or should be interpreted as partial tetanus is not clear. Since it is generally agreed that the arthropod heart is capable of being tetanized, the marked effects seen in Figures 1, 2, and 3 may properly be termed "tetanic effects."

These effects of a relatively high concentration of adrenalin are very different from the effects produced by high concentrations of acetylcholine, thereby indicating that, even though they both have excitatory actions, they act in quite different ways.

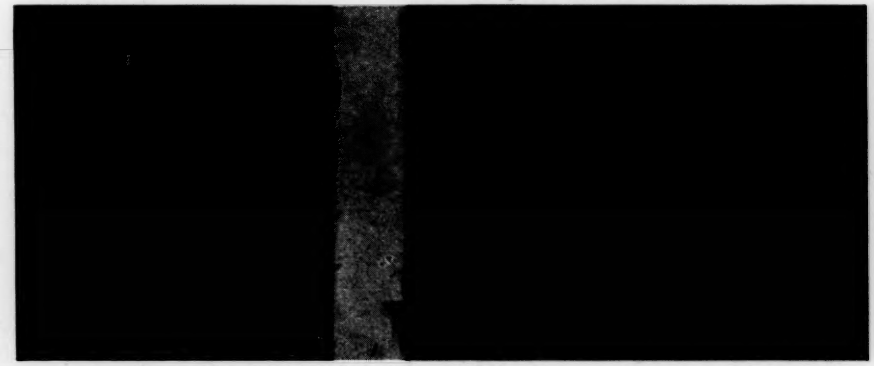

FIG. 4.—Record of the effect of adrenalin 10^{-5}. A section of the record covering a period of 4 minutes has been removed. During this period there were a few weak contractions.

THE INNERVATION OF THE HEART OF *Panulirus*

Fortunately, many features of the innervation of the decapod heart are well known, especially from the excellent work of Alexandrowicz (1932). The situation in the heart of *Panulirus argus* is almost identical with that in the closely related *Palinurus vulgaris* Latreille, which has been worked out in some detail by Alexandrowicz. By using a staining method essentially like his, it was possible to demonstrate the major features of the local nervous system of the heart. These are shown schematically in Figure 5. Unlike *Limulus* and certain other arthropods, the heart ganglion of the decapods is on the inner surface; and in order to expose it, the heart must be removed and split along the ventral side. After suitable staining, one then sees, in *Panulirus*, the view which is shown in Figure 5. Certain regions of the trunk are crossed by muscles, but the major portion is directly exposed. As in *Palinurus* and in all other marine decapods which have been studied, there are five large and four small ganglion cells. Four of the five large cells lie in a mid-dorsal region quite close to one another; the fifth is at some distance posteriorly; while the four small cells are located in the posterior portion of the trunk. In a mid-region of the trunk nine fibers may be seen, and these are grouped into two sizes—five large and four small fibers—corresponding with the two sizes of cell bodies.

Carlson (1905–6) described and figured two separate pairs of nerves arising in the thoracic ganglion of *Palinurus* and innervating the heart. He also demonstrated the effects of stimulating the heart by way of these nerves and found one pair to be inhibitory and the other acceleratory in function. Alexandrowicz could find only a single pair of nerves running to the heart ganglion of this same form, but they contained fibers of two sizes, suggesting a difference in function. The question of the actual number of extrinsic nerves to the heart ganglion remains to be determined, but the observations by Carlson on the functional activity of the extrinsic fibers have been abundantly confirmed.

Certain details regarding the intimate relations between the several ganglion cells remain to be worked out; but Alexandrowicz has shown that, in spite of the small number of nervous elements, the morphological situation in the decapod heart is very complex, and it follows that the functional situation is likewise complex. Transection of the nerve trunk in a mid-region stops the regular beat of the heart. The ganglion may then be considered the pacemaker for the heart, as in *Limulus*, which has been so extensively studied.

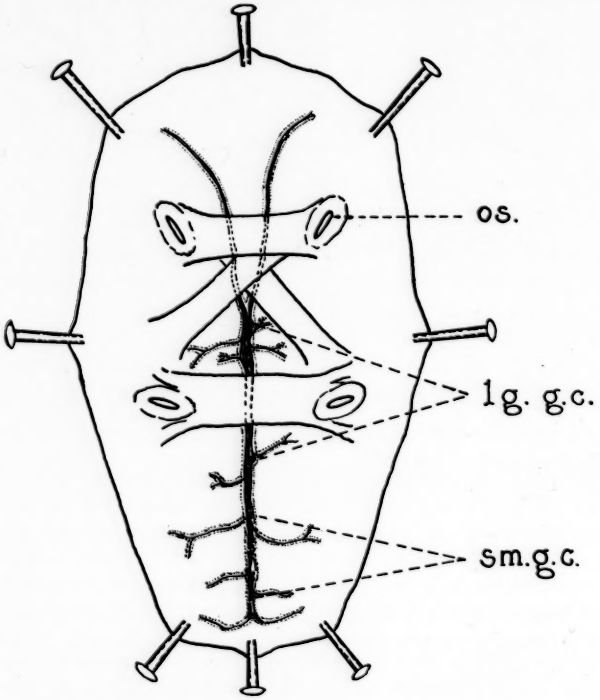

FIG. 5.—View of the heart opened along the ventral side and pinned out to show the nerve trunk and ganglion cells, as seen after staining with methylene blue. Four muscles which run between the ostia (*os.*) cross the heart ganglion. The five large ganglion cells (*lg. g. c.*) are shown with disproportionately large cell bodies. The four small ganglion cells (*sm. g. c.*) are situated toward the posterior end of the heart.

One of the interesting questions regarding pacemaker activity in the decapod heart is the role which the contracting muscle may play in regulating the discharge from pacemaker cells. Alexandrowicz (1932) presents some evidence indicating a return connection from muscles to ganglion cells by way of "dendrites" and suggests that "the impulses conveyed by the dendrites from the muscles would serve for the self-regulation of the rhythmical action of the neuromuscular apparatus of the heart." If such a situation as this does exist in the decapod heart, then any substance which (1) affected the pacemaker activity of

the ganglion cells, (2) facilitated transmission at myoneural junctions, or (3) acted on muscle cells directly might modify the frequency and amplitude of beat. If the local nervous system of the decapod heart is cholinergic, as seems to be the case, then perfused acetylcholine would probably have its greatest effect at myoneural junctions. Adrenalin, on the other hand, might act directly on the muscle cells. However, these are only suggestions, and added information will be necessary before final conclusions can be reached.

SUMMARY

1. Both acetylcholine and adrenalin have an excitatory action on the isolated heart of *Panulirus argus*, the spiny lobster.

2. The actions of eserine and atropine are such as to indicate that the local nervous system of this heart is cholinergic.

3. Low concentrations of acetylcholine cause an increase in frequency and amplitude of beat. High concentrations produce partial or complete tetanus and systolic stoppage.

4. High concentrations of adrenalin produce an increase in frequency and amplitude of beat and occasionally diastolic stoppage but not the tonus changes and tetanus produced by acetylcholine.

5. The different responses to acetylcholine and adrenalin suggest that these substances act on different mechanisms within the heart.

6. The number and arrangement of the ganglion cells and nerve fibers in the heart of *Panulirus* is similar to that in the majority of decapod crustaceans. Five large and four small cells have complex anatomical and physiological relations to one another and to the muscles.

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ON THE KILLING ACTION OF OPTICALLY ISOMERIC NICOTINES IN RELATION TO PROBLEMS OF EVOLUTION OF THE NERVOUS SYSTEM IN ANIMALS

(Ten figures)

G. F. GAUSE AND N. P. SMARAGDOVA
Institute of Zoölogy, University of Moscow

I. INTRODUCTION

IN THE present work the analysis of the killing action of optically isomeric nicotines on different animals was used as a method permitting a new approach to the study of evolution of their nervous systems. The investigation was carried out with vertebrates and with different groups of both fresh-water and marine invertebrates; experiments with the latter were made at the Karadag Biological Station on the Black Sea.

In the first place, certain questions of method arise. Pictet and Rotschy for the first time prepared pure dextrorotatory (unnatural) nicotine in 1904, tested its toxicity on rabbits and guinea pigs, and ascertained that it was less efficient than the natural *l*-nicotine. At the same time, they expressed the view, which subsequently was adopted by a number of compilators, that the mechanism of the toxic action of the dextro-isomer might be different from that of the laevo form, as the symptoms of poisoning with different isomers of nicotine are different. It is evident that, in order to confirm or to reject this hypothesis, exact quantitative investigations are necessary, which were not carried out by Pictet and Rotschy at the time of their first preliminary observations. The necessity of such investigations with optically isomeric nicotines was pointed out by Cushny (1926), and more recently Macht (1929) published a paper on the pharmacologic synergism of stereoisomeric nicotines. The conclusion reached by him is that "an individual cell may possess receptor groups of a laevo and dextro type, and a mixture of two stereoisomers would thus have a double point of attack in place of a single one in case only one of the optic isomers was used." This important conclusion is, however, based on very few observations, which were not treated statistically, from which the author infers that the toxic action of the mixture of *l*- and *dl*-nicotines proves to be stronger than the simple additive action of these isomers. Statistically this conclusion does not hold good because of the very small number of experiments and because of the fact that the author did not attempt to obtain a concentration-toxicity curve which would permit quantitative calculations. Moreover, the preparation of *dl*-nicotine is little suitable for such experiments, as it usually contains an admixture of hydronicotine, which influences its physiological effect (Gause, 1936). Therefore, only the purest dextro-nicotine, obtained by repeated recrystallizations with laevotartaric acid, must be employed here.

The results of our experiments do not give any indications of the existence of any basic differences in the actual mechanism of toxic action of the optic isomers of nicotine. The dextro-isomer, in accordance with the data of Pictet and Rotschy, proves to be less poisonous for vertebrates than the laevo form; but the dynamics of increase of toxicity with concentration is identical in both, and consequently they seem to act on the same link in the system of biological processes but with different speeds in connection with the

spatial factor. This conclusion is also confirmed by the fact that complete identity of temperature characteristics of toxic action of dextro- and laevo-nicotines was observed by us in cold-blooded animals (fishes and tadpoles); such an identity could not exist if the stereoisomers differed as to the mechanism of their action.

This work is based upon the following observations. Higher animals have evidently some specific spatially sensitive substance which is affected by the dextro- and laevo-isomers of nicotine with a difference in rate, the nature of which we shall try to identify exactly. Protozoa do not possess, as our observations have shown, such a spatially sensitive substance; and the dextro- and laevo-isomers of nicotine are equally toxic for them. The question arises: When in evolution did this specific, spatially sensitive substance appear first, and what is its nature? It is interesting to point out that Greenwood, as early as 1890, carried out an extensive comparative investigation of the action of common laevorotatory nicotine on invertebrates, attempting to establish parallelism in the character of the action of this alkaloid which affects, as is known, the nervous systems of animals, with the evolution of their nervous systems. On the basis of purely qualitative observations he concluded:

> The toxic effect of nicotine on any organism is determined mainly by the degree of development of the nervous system. Thus for *Amoeba* it cannot be regarded as exciting or paralysing; it is rather inimical to continued healthy life. As soon as any structural complexity is reached, the action of nicotine is discriminating, and discriminating in such a fashion that the nervous actions which are the expression of automatism, which imply co-ordination of impulses, are stopped first. This is seen dimly in *Hydra*, and it is more pronounced among the Medusae. When structural development goes farther, the selective action of nicotine is traced readily, as for example in *Palaemon*.

Greenwood writes further that "animals which have enough in common to stand near each other in classification may yet react differently upon nicotine, each according to what I may perhaps call its own balance of organisation."

The data obtained by us in a comparative investigation of the toxic action of optically isomeric nicotines differ from those of Greenwood in their greater stability. This is apparently the consequence of the great technical advantages which the work with two optical isomers presents, as compared to the investigation of the action on different animals of a single isomer of some substance. In the latter case we shall never be able to conclude definitely whether the increased sensitiveness to a given preparation in one species of animals is the consequence of the appearance in it of some specific "receptive substance" or whether it results from altered conditions of the penetration of the preparation into the animal, depending upon the form and size of its body, etc. Interpretation of the results is, therefore, possible only in terms of the balance of organization of the animal. But, as experience shows, when we employ two optical isomers identical in all their physicochemical properties, we are able to ascertain the nonspecific differences of sensitiveness of animals to a given substance. In case of such nonspecific differences the effectiveness of dextro- and laevo-isomers differs in equal degree, and the relation between their toxicities, or the "spatial coefficient" of the toxic action, does not change. In case of alterations in the receptive substance, however, we immediately detect alteration in the spatial coefficient of the toxic action.[1]

[1] These considerations hold true for experiments with optically isomeric nicotines, and a more general theory of the principles of such an "asymmetric analysis" of the structure of living systems is reserved for further publications.

Experiments have shown that the methods applied by us permit us to detect either the "absence" or the "presence" of the specific receptive substance spatially sensitive to nicotine, which first appears in the nervous systems of higher worms and which does not undergo any considerable changes in chemical nature in the course of further evolution of animals. Investigation of the presence of this substance in different groups of invertebrates sheds some light upon certain problems of their phylogeny.

II. METHODS

Special attention was given to the purity of preparations used. We employed *l*-nicotine *purissimus* from the Schuchardt Company (Görlitz) with specific rotation attaining $[\alpha]_D = -169°$. The dextro-nicotine was prepared according to Pictet and Rotschy (1904). At first the laevo-nicotine was racemized by heating nicotine sulphate, and then the dextro-isomer was removed from the racemic mixture and purified by repeated crystallization with laevotartaric acid obtained from Schuchardt. Specific rotation of our specimen of dextro-nicotine attains $[\alpha]_D = +140°$.

Before each set of experiments a 10 per cent solution of nicotine in bidistilled water was prepared, then neutralized by 10 per cent HCl to pH = 8.5, and was usually also tested (in suitable dilution) on paramecia (*Paramecium caudatum*) in order to make sure that dextro- and laevo-isomers have quite equal toxicity. From this initial solution the various dilutions required were prepared—for fresh-water animals with bidistilled water, for marine animals with water of the Black Sea.

III. EXPERIMENTS ON VERTEBRATES

a) *Bird* (*Acanthis flammea*).—We made injections of a neutralized solution of nicotine (0.1–0.3 per cent) into the breast muscle of *A. flammea* in quantities of a definite number of milligrams for a hundred grams of weight.

Figure 1 represents the relation of the killing time in seconds to the concentration of the introduced nicotine. It is to be pointed out that for calculation of the relative efficiency of optically isomeric nicotines one should not compare their effectiveness with any arbitrarily chosen concentration of the poison. Thus, for example, with 3 mg. of nicotine for 100 gm. of weight, *d*- and *l*-isomers, as far as their toxicity is concerned, are very near one another, while with 2 mg. of nicotine the difference between them is very distinct. It is therefore obvious that for comparison of the physiological effect of optic isomers we have to employ characteristics of corresponding curves of toxicity which will not depend on the absolute concentration of the poison. The most convenient procedure is to take the constant n, which characterizes the minimal lethal concentration of the poison. In *A. flammea* n equals 0.8 mg. for *l*-nicotine, while for *d*-nicotine n equals 2.5 mg. The spatial coefficient of the toxic action (α), representing the ratio of minimal lethal concentration of *d*-isomer to minimal lethal concentration of *l*-isomer, is 3.12.

In order to get an idea of the dynamics of the toxic action, the curves of toxicity should be converted into straight lines, by plotting the logarithms of the killing time ($\log y$) opposite the logarithms of the corresponding effective concentrations of the poison [$\log (x - n)$]. According to Figure 2, calculated on the basis of ordinates taken from the continuous curves of Figure 1, the slopes of the straight lines, characterizing the dynamics of the increase of toxicity with concentration, are identical for both optic isomers of nicotine; consequently, we have some right to assume the identity of the mecha-

nism of the toxic action of optically isomeric nicotines. In our experiments we could not discover any distinct difference in the symptoms of poisoning with the two isomers.

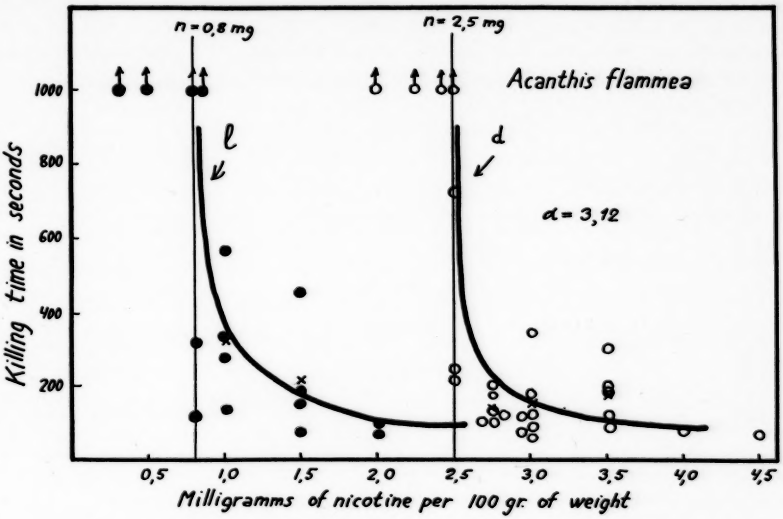

FIG. 1.—Toxic action of optically isomeric nicotines on *Acanthis flammea*

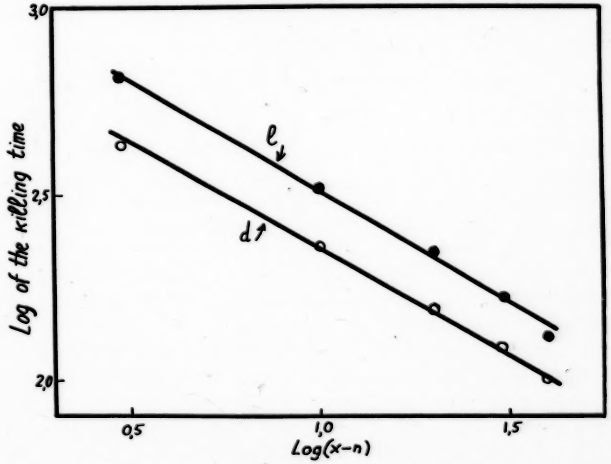

FIG. 2.—Logarithmic curves of toxicity of optically isomeric nicotines for *Acanthis flammea*

b) *Lizard* (*Lacerta viridis*).—The injection of the solution of nicotine was made into the muscle of the right hip. The data obtained are given in Figure 3. In consequence of

great variability of separate observations it is necessary to make here an exact calculation of minimal lethal concentrations of the poison for both optic isomers (threshold values of n). The threshold represents a concentration of poison below which less than 50 per cent of the individuals perish and above which over 50 per cent perish. For every concentration of nicotine the percentage of individuals killed was computed. On the basis of the curve relating the percentage killed to the concentration of the poison, the abscissa (concentration of poison) was determined graphically when the ordinate showed that 50 per cent had perished. Thus, thresholds of poisoning were found to be: for the laevo-isomer, 5.6 mg., and for the dextro-isomer, 13.5 mg., per 100 gm. of weight. The coefficient a is here 2.41.

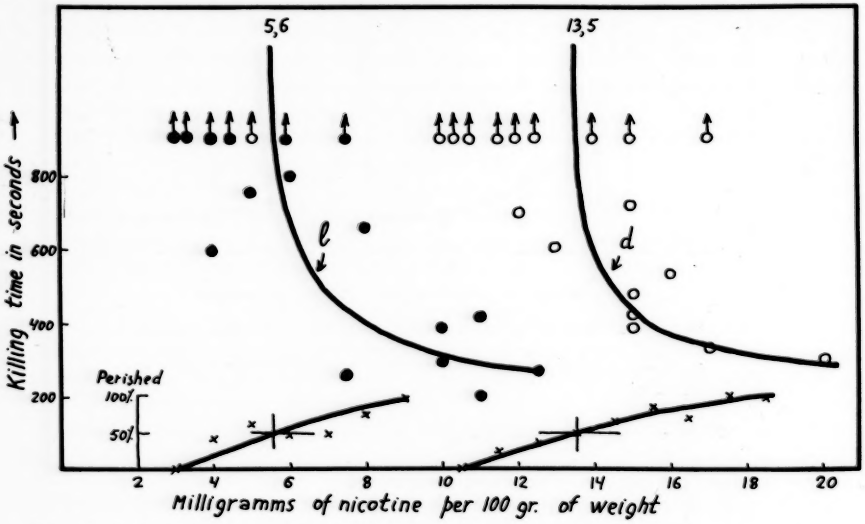

FIG. 3.—Toxic action of optically isomeric nicotines on *Lacerta viridis*. Upper curves refer to killing time in seconds; lower curves refer to percentage of perished individuals.

c) *Tadpoles* (*Rana temporaria*).—Experiments were made with tadpoles (13 mm. long) reared in the laboratory from eggs laid in the open. Tadpoles were placed in neutralized solutions of nicotine of different concentration, prepared with bidistilled water. Each point on the curve of toxicity traced in Figure 4 represents the mean of five observations. The threshold concentrations of poison, as shown in Figure 4, are here very low; and for their exact calculation we have employed the following method. Treating independently the curves of toxicity for the dextro- and laevo-isomers, we at first determined the approximate values of n on the basis of experimental data. Then, working with logarithmic curves, n was selected more exactly, so as to obtain a strictly linear relation between $\log y$ and $\log (x - n)$. In this procedure we evidently implied that the divergence from the linear type of relation, which we are correcting, is due to the incorrectly selected parameter n, but that the nature of the relation of the toxicity to concentration is actually described by the empiric curve of the type $y = k/(x - n)^m$. This as-

sumption follows from the whole experience of modern quantitative toxicology, and with the appropriate selection of parameter n we have actually always obtained a linear relation between log y and log $(x - n)$.

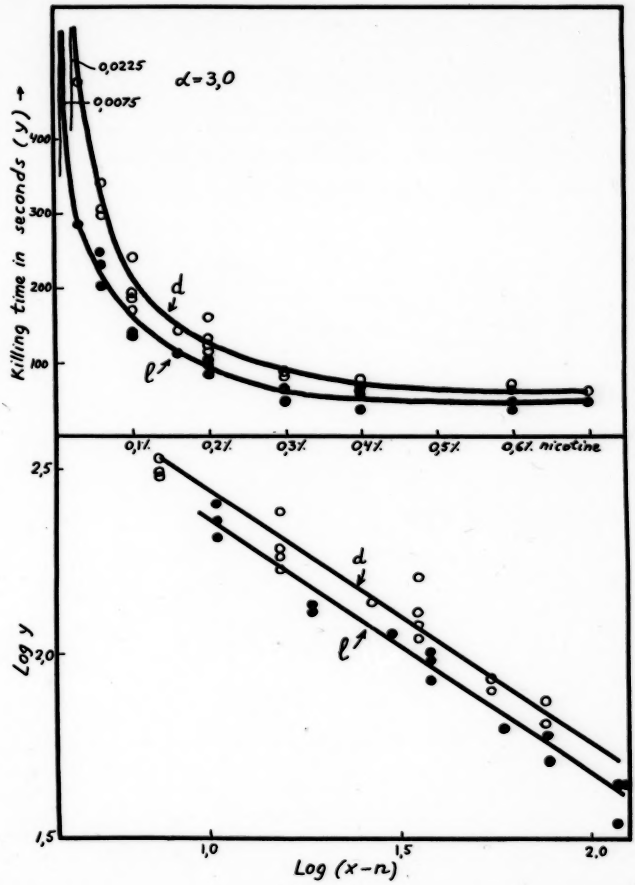

FIG. 4.—Toxic action of optically isomeric nicotines on tadpoles (*Rana temporaria*). Lower curves are logarithmic.

Figure 4 shows that the threshold concentration of the *l*-isomer is 0.0075 per cent and that of the *d*-isomer is 0.0225 per cent. Here the coefficient α equals 3.0. It is very important to point out that the relative position of the curves of toxicity of optic isomers in tadpoles differs sharply at first sight from that observed in lizards. Owing to very small absolute values of threshold concentrations, the curves of toxicity of optic isomers in

tadpoles seem very much approximated, as compared to the relative position of these curves in the lizard. However, the relation between thresholds which finds its expression in the spatial coefficient of the toxic action of nicotine (α) proves to be practically equal in all animals heretofore examined. In conclusion it may be added that in tadpoles the slopes of the logarithmic curves of toxicity are identical in both optically isomeric nicotines.

We may now pass to temperature coefficients of toxic action of optic isomers of nicotine upon tadpoles. The experiments consisted in placing 0.1 per cent solution of nico-

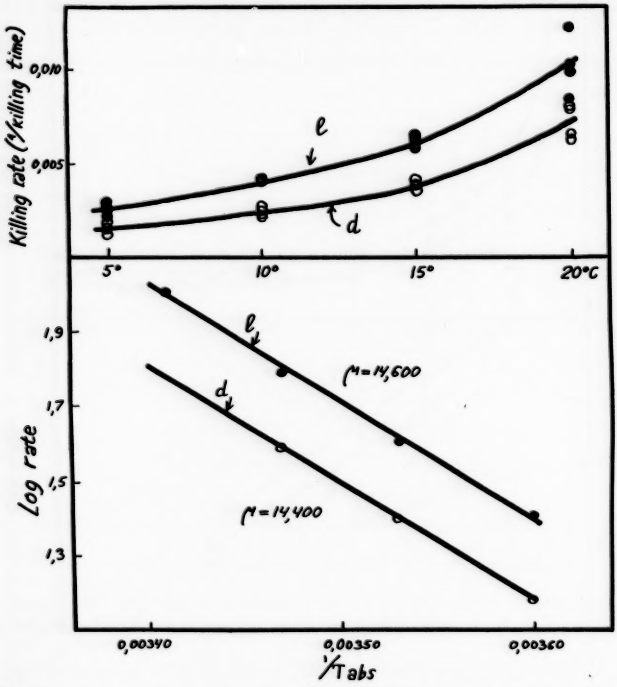

FIG. 5.—The effect of temperature on toxic action of optically isomeric nicotines upon tadpoles (*Rana temporaria*).

tine in experimental vessels in a water bath with controlled temperature. After temperature equilibrium was attained, tadpoles were placed in the solution of nicotine, and the killing time at different constant temperatures was observed. Figure 5 represents the results of 240 experiments of this kind. The upper curves show the relation of killing-rates (values inverse to corresponding killing times in seconds) to temperature. For the purpose of computation of temperature characteristics of toxic action we plotted on the lower curves of Figure 5 logarithms of the killing-rates against the inverse values of absolute temperatures. When computing logarithms of killing-rates, we made use of the mean values of speeds for each temperature. It may be stated definitely that temperature charac-

teristics of toxic action practically coincide for the laevo- (μ = 14,600) and dextro- (μ = 14,400) isomers of nicotine. Thus, the relation of toxic action to temperature strongly supports the view that the mechanism of toxic action is identical in optically isomeric nicotines.

d) *Fish* (*Leuciscus idus* var. *orfus*).—Experiments with brood of *L. idus* were made according to the same plan as with tadpoles. Each point on Figure 6 represents the mean

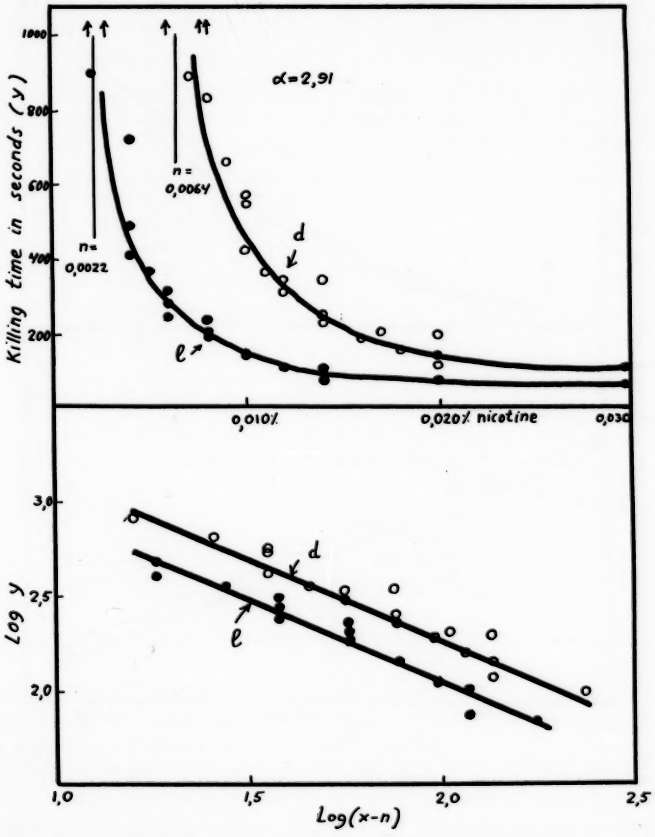

FIG. 6.—Toxic action of optically isomeric nicotines on *Leuciscus idus*. Lower curves are logarithmic

of five observations. It may be pointed out that the spatial coefficient of toxic action of nicotine (α) here equals 2.91. Slopes of logarithmic curves of toxicity coincide in optical antipodes.

Figure 7 represents the results of experiments on temperature characteristics of the toxic action of isomers of nicotine. For these experiments practically isotoxic concentrations of antipodes were selected, i.e., *l*-nicotine in the concentration 0.007 per cent and *d*-nicotine in the concentration 0.014 per cent. Altogether, 120 such experiments were

made. Their results, according to Figure 7, demonstrate the identity of temperature characteristics of intoxication of *L. idus* with dextro- and laevo-isomers of nicotine.[2]

e) *Fish* (*Lebistes reticulatus*).—The last representative of vertebrates studied by us was the brood of *L. reticulatus*. Each point on Figure 8 is the mean of three observations. Coefficient α here equals 2.4.

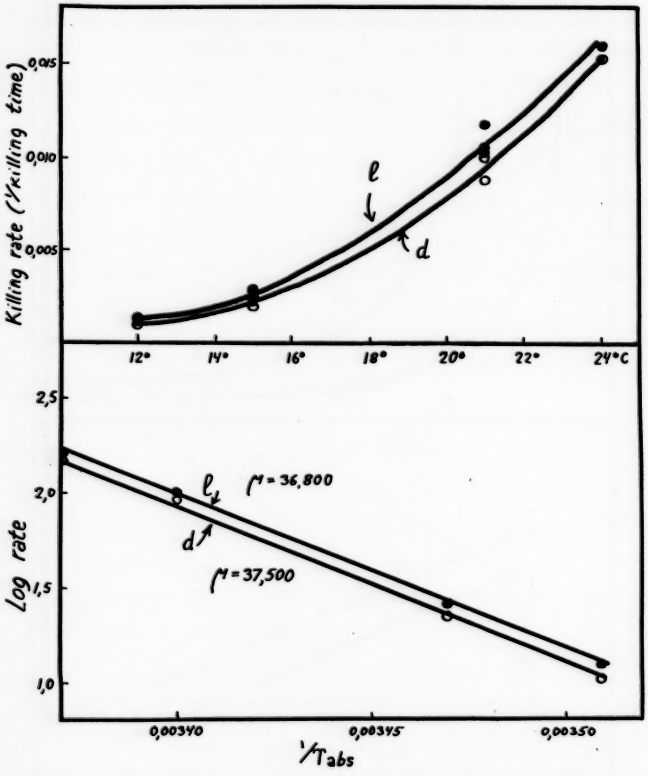

FIG. 7.—The effect of temperature on toxic action of optically isomeric nicotines upon *Leuciscus idus*

f) *Conclusions*.—Experiments on vertebrates permit us to make the following two essential conclusions. First, the mechanism of toxic action of dextro- and laevo-isomers of nicotine prove to be equal. This is demonstrated by the identity of dynamics of toxic action (identity in slopes of logarithmic curves of toxicity) and the identity of temperature characteristics of toxic action in optically isomeric nicotines. Second, spatial co-

[2] We cannot, as yet, give any explanation of the fact that the temperature characteristics of intoxication in tadpoles are of the order of 14,000 and that in the *L. idus* of the order of 37,000.

efficients of the toxic action of nicotine prove to be of one and the same order of magnitude in all investigated animals:

Acanthis flammea	$\alpha = 3.1$
Lacerta viridis	$\alpha = 2.4$
Rana temporaria	$\alpha = 3.0$
Leuciscus idus	$\alpha = 2.9$
Lebistes reticulatus	$\alpha = 2.4$
Mean	$\alpha = 2.8$

The mean value of this coefficient is 2.8. The first and the second conclusions, being independent, agree well with one another. Inasmuch as the mechanism of toxic action is identical in optically isomeric nicotines, we can judge, by the difference of their effects,

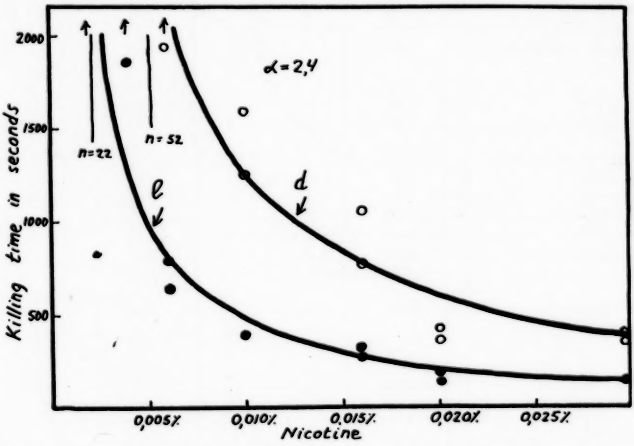

FIG. 8.—Toxic action of optically isomeric nicotines on *Lebistes reticulatus*

the spatial properties of some specific receptive substance; as the difference of effects remains constant, one can conclude that the chemical nature of the receptive substance within the vertebrates remains also essentially constant.

This last result is of some interest in connection with the method of investigation used. It represents an experimental proof of the fact that the conditions of the penetration of nicotine into the animal do not affect significantly the value of the spatial coefficient. Nicotine was introduced into the muscle of *Lacerta* while *Lebistes* were immersed in the solution of nicotine; nevertheless, the spatial coefficients of toxic action of nicotine are identical in these two animals. Absolute sensitiveness to nicotine in *Acanthis* is considerably higher than that in *Lacerta* (0.8 mg. per 100 gm. of weight, as compared to 5.6 mg. per 100 gm. of weight), but practically it does not influence the spatial coefficient of the action of nicotine. It is therefore evident that the spatial coefficient (α) gives us an opportunity to judge of the chemical nature of the specific receptive substance.

IV. EXPERIMENTS ON INVERTEBRATES

The results of experiments with fresh-water and marine invertebrates are given in Table 1. For each species we recorded specific symptoms of intoxication (taking into consideration the moment of the cessation of voluntary movements) and plotted curves

TABLE 1

TOXIC ACTION OF OPTICALLY ISOMERIC NICOTINES UPON INVERTEBRATES

Animal	Fresh Water or Marine	Range of Concentrations Studied (Percentage)	No. of Experiments	Results	
Protozoa:					
1. *Paramecium caudatum*	f.w.	0.6 – 1.2	80		
2. *Euplotes patella*	f.w.	1.0 – 2.6	34		
3. *Stentor coeruleus*	f.w.	0.4 – 0.8	24		
4. *Spirostomum ambiguum*	f.w.	0.2 – 0.6	24		
Coelenterata:					
5. *Hydra fusca*	f.w.	1.0 – 2.0	24		
6. *Cladonema radiatum*	mar.	0.2 – 0.5	12		
Platyhelminthes, Turbellaria:					
7. *Polycelis nigra*	f.w.	0.3 – 0.8	34	*d*- and *l*-nicotines are isotoxic	
8. *Phaenocora* sp.	f.w.	0.2 – 1.0	32		
9. *Dalyella brevimana*	f.w.	0.4 – 1.0	30		
10. *Procerodes lobata*	mar.	0.3 – 0.8	10		
11. *Leptoplana tremellaris*	mar.	0.01 – 0.05	16		
Rotatoria:					
12. *Euchlanis triquetra*	f.w.	0.02 – 0.1	20		
13. *Rotifer vulgaris*	f.w.	0.06 – 0.2	24		
Nemertini:					
14. *Lineus lacteus*	mar.	0.4 – 1.0	12		
Annelida:					
15. *Saccocirrus papillocercus*	mar.	0.1 – 0.7	19		$a = 2.30$
16. *Perinereis cultrifera*	mar.	0.3 – 1.0	18		$a = 1.90$
17. *Arenicola grubii*	mar.	0.75	8		.....
18. *Pristina longiseta*	f.w.	0.1 – 1.0	38		$a = 2.09$
19. *Limnodrilus hoffmeisteri*	f.w.	0.025 – 1.6	136		$a = 3.45$
20. *Helobdella stagnalis*	f.w.	0.0002– 0.007	78		$a = 4.00$
21. *Nais communis*	f.w.	0.1 – 0.6	60	*l*-nicotine is more toxic	$a = 2.41$
22. *Chaetogaster langi*	f.w.	0.02 – 0.2	54		$a = 3.13$
23. *Stylaria lacustris*	f.w.	0.3 – 0.6	20		.....
24. *Aelosoma variegatum*	f.w.	0.2 – 0.6	62		$a = 1.76$
25. *A. hemprichi*	f.w.	0.2 – 0.6	58		$a = 1.84$
Chaetognatha:					
26. *Sagitta setosa*	mar.	0.025 – 0.10	16		$a = 2.7$
Arthropoda:					
27. *Daphnia magna*	f.w.	1.2 –10.0	124		
28. *Cyclops serrulatus*	f.w.	0.25 – 1.0	54		
29. *Gammarus marinus*	mar.	0.8 – 1.0	10	*d*- and *l*-nicotines are isotoxic	
30. *Drosophila melanogaster* (two-day-old larvae were immersed in nicotine solutions)	.....	0.4 – 1.0	30		

relating killing time in seconds to the corresponding concentration of nicotine. A part of these curves is represented on Figures 9 and 10.

Table 1 shows that all the invertebrates examined by us can be divided into two distinct groups. For those belonging to the first group the dextro- and laevo-isomers of nicotine are equally toxic, and the curves of toxicity of optical antipodes fully coincide with one another (Fig. 9). For the members of the second group the laevo-isomer of nicotine is more toxic than the dextro-isomer (Fig. 10). The one or the other attitude toward opti-

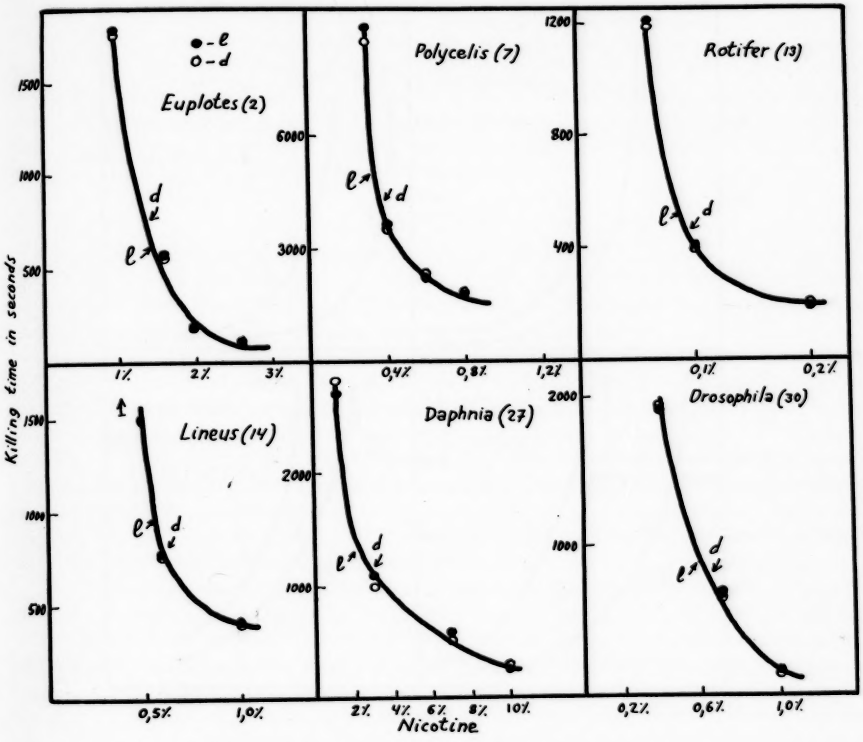

FIG. 9.—Toxic action of optically isomeric nicotines upon some invertebrates

cally isomeric nicotines appears to be specific for the large systematic groups of animals, and within these groups does not disclose any variations.

Examining Table 1 more closely, we see that all the representatives of Protozoa, Coelenterata, Turbellaria, Rotatoria, and Nemertini studied by us belong to the first group of animals. It means that they are devoid of a spatially specific receptive substance to intoxication by nicotine. It is noteworthy that in invertebrates the spatial coefficient of the action of antipodes is not affected by the differences in the absolute sensitiveness to nicotine peculiar to separate species, as has been already recorded for verte-

brates. Thus, for example, *Leptoplana* is considerably more sensitive to nicotine than *Procerodes*, but both these turbellarians are characterized by equal effect of dextro- and laevo-isomers of nicotine. The number of such examples could be considerably augmented.

A stronger effect of the laevo-isomer of nicotine is met with for the first time in Annelida, and remarkably in Archiannelida (*Saccocirrus*), Polychaeta, and Oligochaeta. Then it is observed in primitive representatives of Deuterostomia (*Sagitta setosa*). In Arthropoda

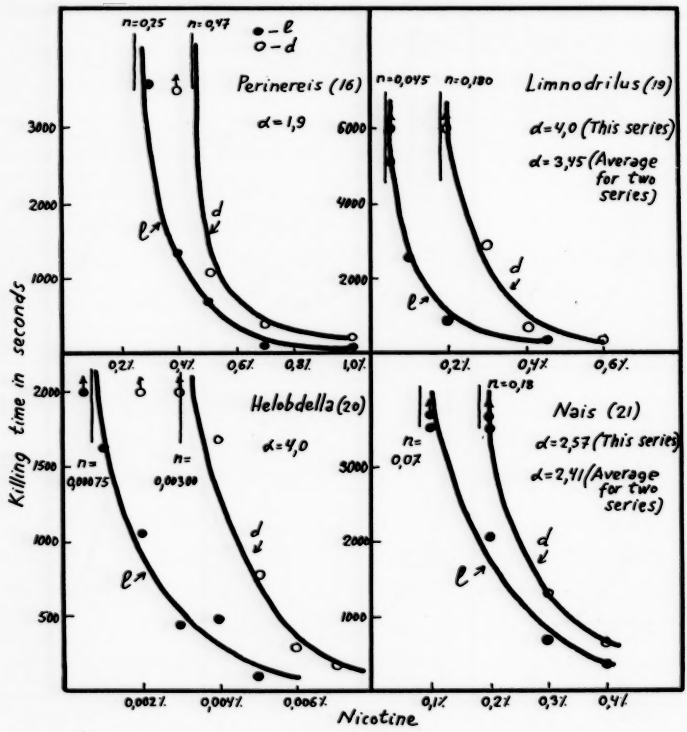

FIG. 10.—Toxic action of optically isomeric nicotines upon some invertebrates

(Crustacea and Insecta) this effect is absent, and here again we find equal toxicity of dextro- and laevo-isomers of nicotine.

Let us now turn to the following question: Is the excess of the efficiency of laevo-isomer over that of the dextro-isomer, in those invertebrates possessing the spatial effect, of the same order of magnitude as in the vertebrates? For invertebrates we have:

Saccocirrus papillocercus ..... $\alpha = 2.3$
Perinereis cultrifera ..... $\alpha = 1.9$
Pristina longiseta ..... $\alpha = 2.1$

Limnodrilus hoffmeisteri	$\alpha = 3.4$
Helobdella stagnalis	$\alpha = 4.0$
Nais communis	$\alpha = 2.4$
Chaetogaster langi	$\alpha = 3.1$
Aelosoma variegatum	$\alpha = 1.8$
A. hemprichi	$\alpha = 1.8$
Saqitta setosa	$\alpha = 2.7$
Mean	$\alpha = 2.6$

When considering these figures, one must have in view that inaccuracies in determining the value of the coefficient α may be considerable. Thus, in case of two independent determinations we found: in *Nais*, 2.57 and 2.25; in *Limnodrilus*, 2.9 and 4.0; in *Aelosoma variegatum*, 1.79 and 1.74; and in *Aelosoma hemprichi*, 1.50 and 2.18. In the light of these observations we may infer that the average coefficient α in invertebrates (2.6) and in vertebrates (2.8) is a magnitude of the same order.

V. DISCUSSION

The data obtained by us show that in Protozoa, Coelenterata, Turbellaria, Rotatoria, and Nemertini the spatially specific receptive substance affected by nicotine is absent; both optical isomers are here equally toxic. Annelida, Chaetognatha, and vertebrates possess the spatial receptor, while in Arthropoda it is again absent.

In order to try to comprehend the nature of the receptive substance we have to consider several groups of facts. First of all, identity of the spatial coefficient and, consequently, identity of the receptive substance is observed in invertebrates and in vertebrates in spite of essential differences in morphology of their nervous systems, which, as it is known, is affected by nicotine. Consequently, we have to interpret the receptive substance as some widely spread chemical constituent. However, this chemical constituent is not an obligatory component of every nervous system. This receptive substance is absent from the nervous systems of lower invertebrates (Turbellaria and Nemertini), although they show considerable differentiation.

The next step toward understanding the nature of the receptive substance may be made by examining current views concerning the mechanism of nicotine intoxication. In a number of papers Thomas and Franke (1924, 1928, 1933) have shown that paralysis of peripheral neuromuscular junctions of respiratory muscles is the cause of death of higher animals in acute nicotine poisoning. This view was confirmed by Gold and Brown (1935). The action of nicotine upon neuromuscular junctions brings us directly to the classical observations of Langley (1905), who found that in the system "neuro-muscular junction" there is certain sensitive "receptive substance" which is the first to be affected by nicotine.

Let us try to formulate a hypothesis of the possible nature of the "receptive substance." Since the classical works of Loewi, it is known that there is a chemical step in the transmission of impulses from nerves to effectors according to the scheme: (1) nerve impulse → (2) chemical mediator → (3) receptive substance → (4) specific response (for literature see Cannon and Rosenblueth, 1937). There are some indications that the chemical mediator of voluntary muscles of higher animals is acetylcholine; and its action on the receptive substance of the effector in this case reminds one of the action of nico-

tine: in small doses it excites, and in large doses paralyzes.[3] According to current views, nicotine, in acute poisoning of the animal, affects in some irreversible way the receptive substance, so that acetylcholine mediation cannot act any more.

All these suggestions can be expressed more definitely in the following way. Nicotine (at least in experiments of our type) acts upon neuro-effector synapses of voluntary muscles. In its action it reminds one of acetylcholine, a substance transmitting excitement in these synapses. Consequently, the receptive substance in nicotine poisoning must have some close relation to the receptive substance for chemical mediation. Our experiments permit us to divide animals into two groups, differing as regards the nature of the receptive substance affected by nicotine. May it not prove that animals possessing receptive substance structurally active and structurally inactive to nicotine differ from one another in the receptive substance of chemical mediation and, consequently, in the mechanism of transmission of nervous impulses?

In order to answer this question, we have to examine the data of physiologists concerning the distribution of acetylcholine in different groups of invertebrates. In the first place we must point out that the method of identification of acetylcholine is subject to criticism. The presence of acetylcholine is ascertained by the action of the extract under examination on different organs while, as Cannon and Rosenblueth (1937) pointed out, none of these organs is strictly specific and in this way the distinguishing between their reaction to acetylcholine and to other substances become difficult. The results may be regarded as reliable if, with several different methods, quantitatively coinciding data are obtained. This condition, however, is seldom fulfilled. Therefore, communications concerning this problem appearing in the literature must be critically viewed. The most extensive and elaborate investigations were carried out by Bacq (1935) at the Zoölogical Station of Naples. First, he did not discover in the tissues of different coelenterates either acetylcholine or the enzyme destroying it, choline-esterase. The muscles of annelids and of lower Deuterostomia (*Holothuria*) contain acetylcholine and choline-esterase. Highly interesting but yet somewhat contradictory are the data concerning Crustacea and Insecta. In the muscles of Crustacea was found only a small quantity of acetylcholine. Hence, Bacq inferred that the transmission of impulses from the motor nerve to the muscle in Crustacea is not accomplished by means of acetylcholine mediation. He insisted upon this at the conference devoted to this problem in Cambridge, England, in June, 1937. On the other hand, there are some preliminary communications concerning the presence of choline-esterase in ganglia of Crustacea (Nachmansohn, 1937). However, neuro-effector synapses of the muscles of Crustacea are not typical acetylcholine systems, if only for the reason that they are extremely insensitive to external acetylcholine.

In comparing Bacq's observations with our results (see Table 2), it is quite apparent that the optically active receptor for nicotine poisoning is found where classical acetylcholine mediation is observed and is absent where the classical acetylcholine mediation is not detected. In six cases we have to do with a complete coincidence of these two series of independently obtained results. If our suggestions are correct, the spatial effect of

[3] A parallelism in the action of nicotine and acetylcholine can be observed on our experimental material. It is known that the leech is extremely sensitive to acetylcholine and reacts to insignificant traces of it (Minz, 1932). According to Table 1, the leech, *Helobdella stagnalis*, stands out sharply from all other animals by its extreme sensitiveness to nicotine, which proves to be toxic in concentration 0.00075 per cent.

nicotine could be employed to detect the presence of the specific receptor characteristic for the acetylcholine system in the neuro-effector synapse of voluntary muscles.

In conclusion we must examine the data obtained from the point of view of some problems of the phylogeny of invertebrates. If one agrees to consider the presence of spatial effect in nicotine poisoning and the presence of the typical acetylcholine system in neuro-effector synapses of voluntary muscles as equivalents, the following general picture may be sketched. In Protozoa, Coelenterata, Turbellaria, and related groups (Rotatoria and Nemertini) the acetylcholine system has not yet appeared. The similarity in this respect of Nemertini to Turbellaria may be considered as another proof in favor of the origin of Nemertini from turbellarian ancestors. The acetylcholine system appears for the first time in annelids (it is already present in archiannelids) and remains through lower Deuterostomia (Chaetognatha and *Holothuria*) up to vertebrates. Arthropoda, evidently, have secondarily modified their acetylcholine system.

TABLE 2

DISTRIBUTION OF THE ACETYLCHOLINE MEDIATION AND OF THE SPATIAL EFFECT IN POISONING WITH OPTICALLY ISOMERIC NICOTINES

Groups of Animals	Spatial Effect in Poisoning with Nicotine (Gause and Smaragdova)	Acetylcholine Mediation (Bacq, 1935)
1. Coelenterata	Absent	Absent
2. Annelida	Present	Present
3. Lower Deuterostomia (*Holothuria* for acetylcholine and Chaetognatha for nicotine)	Present	Present
4. Crustacea	Absent	Absent
5. Insecta	Absent	Absent
6. Vertebrates	Present	Present

To what degree may we make use of the presence of the acetylcholine system in solution of the phylogenetic problem? To this end we shall at first turn to such a substance as haemoglobin, which has several times independently arisen in evolution and evidently cannot serve to establish any phylogenetic relationships. Baldwin (1937) writes:

Haemoglobin appears to be distributed in a very haphazard fashion without the slightest regard to zoölogical classification. It is present in all the vertebrates, in a few holothurians, several crustaceans and at least two insects, in several lamellibranchs and one gastropod (*Planorbis*), in many annelid worms and even in two species of Platyhelminthes. For a long time it was difficult to understand how so many animals of such different kinds can produce haemoglobin, while the ability to produce the other pigments seems to be very sharply confined to particular groups of closely related animals. But it is now known that practically all living cells contain cytochrome, which like haemoglobin itself is a haem pigment, and that the haem of cytochrome is certainly very like that of haemoglobin and possibly even identical with it. Since cells in general are capable of elaborating the haem of cytochrome, it is very probable that they can also produce the haem of haemoglobin if the need arises. In the vertebrates, haemoglobin acts as a respiratory pigment in the ordinary sense, but in the invertebrates it seems to have arisen independently in many different species as an adaptation to longer or shorter periods of oxygen deficiency.

In other words, we may say that for transformation of cytochrome into related haemoglobin a single, or at least a few simple, mutations were sufficient, and these were evidently accomplished and fixed by natural selection in several independent cases.

Returning to the acetylcholine system, one can say that its distribution in animals differs basically from that of haemoglobin. The presence or absence of the acetylcholine system is a characteristic not of separate species but of large systematic groups. It is absent in all turbellarians and is present in all annelids; we have never found an exception to this rule. Through the lower Deuterostomia the acetylcholine system of worms is directly related to the acetylcholine system of vertebrates; thus, we have some right to suggest that acetylcholine system has appeared in evolution only once and has never independently arisen since that time.

The problem of the position in zoölogical classification of lower Deuterostomia which we consider to be a connecting link between worms and vertebrates is still open to question. Dogiel (1934) summarizes in the following manner the arguments of comparative morphology.

> According to one version, Deuterostomia are so isolated from other invertebrates that they have to be derived from Coelenterata independently of worms through some as yet insufficiently known stages. The weak point of this hypothesis is that it demands to accept an excessively sharp transition from coelenterates immediately to the representatives of higher animals possessing coelom, blood-vessel system, etc. According to other views, the roots of Deuterostomia have to be found among higher worms, namely, in the group of Vermidea. Such a conclusion is supported by a number of anatomical facts. However, the difference between Deuterostomia and Vermidea is so great that the origin of the former from the latter has to be considered not quite proved.

The data on distribution of acetylcholine system discussed in this paper suggest in favor of the second hypothesis.

VI. SUMMARY

1. Protozca, Coelenterata, Turbellaria, Rotatoria, Nemertini, and Arthropoda do not possess any spatially specific receptive substance to nicotine; and both optically isomeric nicotines are, for them, equally toxic. For Annelida, Chaetognatha, and Vertebrata the laevo-isomer of nicotine is more efficient than the dextro form, and therefore these animals possess spatially specific receptive substance.

2. In considering different groups of animals, one can record a complete coincidence between the presence and the absence of the spatial effect of nicotine and the presence or absence of the typical acetylcholine system of transmission of nervous impulses. Thus, the spatial effect of nicotine could be employed to detect the presence of the acetylcholine system in neuro-effector synapses of voluntary muscles. These results are discussed in relation to some problems of the phylogeny of invertebrates.

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POSTNATAL DEVELOPMENT OF REPRODUCTIVE SYSTEM IN MALE GUINEA PIGS AND ITS RELATION TO TESTIS HORMONE SECRETION[1]

(One figure)

E. DUANE SAYLES
Department of Zoölogy, University of Chicago, and
Virginia Junior College

A STUDY of the effects of castration on the guinea pig introduced so many questions that had a bearing upon the differentiation of the reproductive system from the postnatal to the mature functional state that it was deemed advisable to investigate the prepuberal development of this system. Such a study in the rat has been carried on by Price (1936), and for the rat testis by Moore (1936). Some of the data obtained from such a study on the guinea pig are reported here, together with some of the effects of prepuberal castration, which should aid in interpreting normal reproductive development. The effects of castration in mature males will be reported elsewhere.

This paper will concern itself with the general growth and histology of the testis and accessory organs of reproduction and with the functional differentiation of the testes, prostate gland, and seminal vesicles; Cowper's gland in the guinea pig has already been studied by Heller (1932). The main points to be stressed are the time at which germ-cell differentiation occurs in the testis and the discharge of spermatozoa in the ejaculate, and the correlation of these events with evident signs of testis hormone secretion, the indications for which should be registered by changes in the accessory organs of reproduction. Effects of castration in the prepuberal males has been of some aid in the principal interpretations of some of these events.

MATERIAL AND METHODS

One hundred and eighty-five animals were utilized in this investigation: they were secured from active breeding colonies at the University of Chicago; for many of the younger animals the writer is indebted to Dr. Sewall Wright. Tissues for fixation were removed from animals immediately after death. The removal of the urethra and annexed parts permitted an adequate separation of the anterior lobe of the prostate from the middle and posterior lobes, which were not separated from each other for purposes of weight records. A torsion balance and chainomatic balance were employed for fresh gland weights. Eight different fluids for fixation and several stains were utilized in preliminary searches for specific cell organs or inclusions that might prove adequate as diagnostic indicators of the action of testis hormone on the accessory organs. Studies of Golgi material were made from preparations by the Ludford modification of the Mann-Kopsch technique (1925, 1926), both the short and long methods being employed. Paraffin sections were utilized throughout. Other details of technique will be indicated in appropriate places.

[1] This investigation has been aided by a grant from the Rockefeller Foundation to the University of Chicago. The author desires to express his appreciation to Dr. Carl R. Moore for his generous help and guidance during the course of the investigation.

GENERAL GROWTH OF REPRODUCTIVE PARTS

The growth tendencies of various parts of the male reproductive tract were determined from tissues collected from 73 animals of known ages ranging from 9 days to

TABLE 1

TABLE OF BODY AND GLAND WEIGHTS

Age (Days)	No. of Cases	Body Weight (Grams)	Weight (Milligrams)				
			Testes	Seminal Vesicles	Vas deferens Epididymis	Prostate Lobes	
						Middle and Posterior	Anterior
9	4	115	100.2	43.4	95.0	35.6	7.0
10	2	149	134.2	105.3	128.4	57.5	23.0
11	1	118	105.4	39.6	85.6	33.0	11.4
12	2	120	79.6	46.0	79.9	34.0	7.7
18	5	128	118.8	44.6	99.3	40.6	10.1
20	1	214	195.4	208.2	159.4	103.0	28.6
21	1	230	274.6	268.4	146.0	112.4	50.0
22	2	146	135.0	60.0	110.2	46.1	13.2
25	1	190	117.4	76.4	124.0	50.0	14.2
27	1	196	197.4	89.6	155.8	74.4	34.0
29	2	208	250.3	226.0	162.5	91.3	35.8
30	2	192	232.1	94.9	96.1	38.7	18.0
31	3	195	236.0	108.1	119.9	46.0	15.3
35	2	266	366.4	425.1	192.4	106.6	59.0
38	1	256	313.4	314.6	198.6	125.8	62.0
42	1	232	205.8	191.6	155.6	103.4	33.4
45	5	245	430.8	226.6	175.2	86.7	40.5
47	2	350	624.0	598.1	241.2	167.9	77.8
49	3	280	509.4	853.3	218.0	184.7	107.6
50	2	300	698.0	984.8	240.1	183.7	111.6
55	1	276	478.4	568.4	190.0	116.4	65.8
56	1	321	758.6	691.8	254.8	197.4	104.6
58	2	279	626.4	692.6	301.5	189.3	72.6
59	1	284	544.0	890.0	273.4	214.2	120.0
61	1	292	634.0	769.8	237.2	195.0	67.0
71*	1	270	242.2	82.8	178.2	63.4	23.8
72	1	308	1128.2	378.4	314.6	166.2	112.0
74	2	362	1389.5	510.0	324.0	222.7	101.2
77	2	360	1418.0	596.1	332.3	178.0	105.9
79	1	240	452.0	143.4	187.2	92.2	31.0
82	1	348	1072.0	430.0	256.6	171.4	78.0
83	3	377	1304.8	557.1	321.2	150.3	139.3
84	4	366	1161.5	673.4	303.2	181.8	102.6
85	2	311	1007.9	415.6	272.4	160.3	87.2
87	1	382	1426.8	437.4	318.2	184.6	87.4
91	1	305	975.7	600.0	243.2	163.6	81.5
98	1	299	1231.1	718.9	305.6	116.5	57.0
99	1	310	905.4	192.3	249.7	93.2	38.6
102	1	294	665.4	267.6	206.3	88.6	51.5
123	1	494	2239.0	1132.8	618.8	276.2	168.4
130	1	464	2025.8	822.2	497.2	241.4	157.0

* All animals including and above this age were ejaculated previous to killing by at least 2 days.

130 days old; by the latter age the male has attained its reproductive function. These data are presented in Table 1 and Figure 1. Seminal vesicles from animals older than

70 days are omitted from the graph, for the reason that all animals of this age and older were subjected to an electrical stimulation to induce an ejaculation; hence, a discrepancy in growth-rate is introduced.

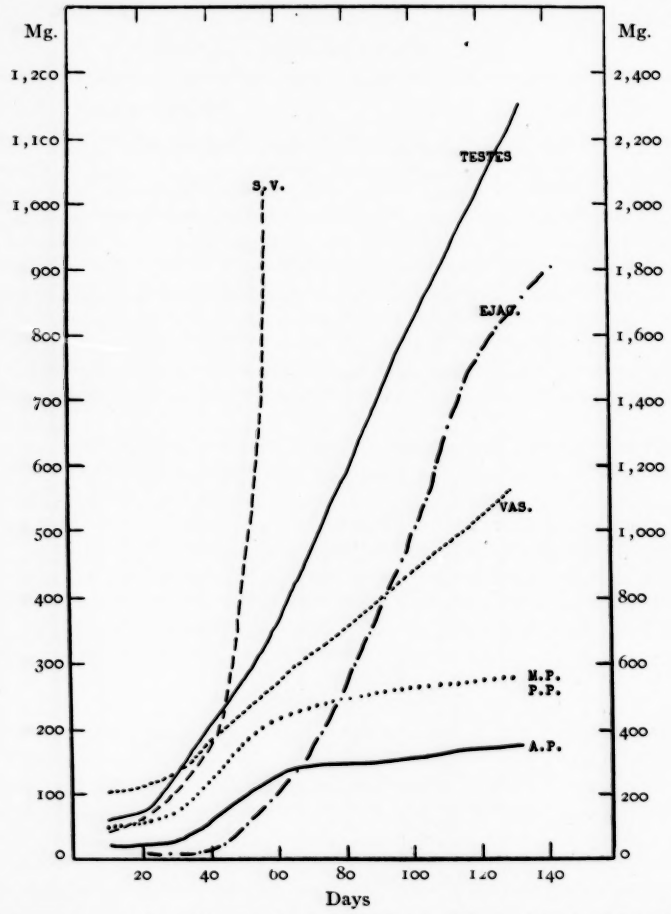

FIG. 1.—Growth curves of reproductive gland weights in normal animals and weights of ejaculates of prepuberal guinea pigs. Curves drawn by inspection from scatter of points of data in Table 1 and from scatter of points of the weights of 244 electrical ejaculates of normal young animals. Testes and ejaculate weights plotted after the right-hand scale; others, after the left-hand scale.

Aside from details of individual growth increments, perhaps the most striking point is the tendency of all organs to show a pronounced acceleration in weight beginning about the thirtieth to fortieth day of life. Testis increases occur slightly earlier than those of the accessory reproductive organs.

Since a detailed comparison of weights of individual accessory organs with general

body-growth shows a decidedly disproportionate growth on the part of the internal organs, one is immediately impressed with the indications of some critical period in the life of the organism at about this period. Testis weight, though increasing at a more rapid rate than general body-growth, exhibits a smoother general growth than the accessory organs; hence, the initiation of, or enhancement of, testis hormone secretion is probably responsible for the rather sudden accelerated growth of these accessory parts.

FUNCTIONAL DEVELOPMENT OF PROSTATE AND SEMINAL VESICLES

Seminal vesicles of most rodents produce a secretion that sets in a firm coagulum when mixed with prostatic secretions, and in the guinea pig the ejaculate forms a coagulated vaginal plug firmly attached to the walls of the vagina. The secretory function of these accessory organs has been shown to be dependent upon the internal secretion of the testes (Camus and Gley, 1896; De Bonis, 1909; Walker, 1910; Moore and Gallagher, 1930).

It was demonstrated by Battelli (1922) that male guinea pigs experience an ejaculation when stimulated on the head by an alternating electric current, and Moore and Gallagher (1930) have employed the technique as a means of studying the influence of testis hormone in the animal. It appeared advisable in this study to utilize the reaction as a means of determining the onset of function of the prostate and seminal vesicles in young animals, which would, in turn, establish at least the period in testis hormone secretion at which the effective threshold of hormone concentration was reached to induce this function.

Fifty-five guinea pigs were stimulated with electric shocks between the ages of 11 and 89 days after birth; and in case the initial stimulation failed to evoke an ejaculation, subsequent stimulations were given at weekly intervals until the first ejaculation was obtained. These stimulations followed the method of Moore and Gallagher and consisted on each occasion of a series of three shocks of 10 seconds each with approximately a minute interval between shocks. For smaller animals an alternating current of 6 volts was sufficiently strong, and graduated doses were utilized until animals of a weight of 350–400 gm. received between 25 and 30 volts. One needle-point electrode was inserted at the base of the skull just dorsal to the foramen magnum, whereas the other blunt terminal was inserted into the mouth. The collected ejaculate was weighed and examined for coagulation and for spermatozoa.

An analysis of the data shows that coagulable ejaculates may be obtained from animals as early as day 21; at this age a male weighing 194 gm. produced a coagulable ejaculate of 10 mg. The majority of males void a coagulable ejaculate by the thirtieth day of life, since 9 of 11 males receiving their first stimulation on day 26 produced coagulable ejaculates; these ejaculates varied from 1 mg. in weight to 150 mg., or an average of 68 mg. One male failed to void an ejaculate until the seventy-fourth day of life, and all stimulations at ages later than this yielded typical coagulable ejaculates. Analysis of the entire data on this point reveals that, among animals receiving stimulations between ages of 21 and 27 days, 45 per cent produced ejaculates; ages 28–34 days, 61 per cent; ages 35–41 days, 71 per cent; ages 42–48 days, 85 per cent; ages 49–55 days, 93 per cent; over 75 days, 100 per cent.

The curve of weight of the ejaculates shown in Figure 1 exhibits a gradual acceleration up to the oldest animal, tested on day 142. The amount of discharge at this time is essentially within the range of normal for adult animals, since Moore and Gallagher

reported 1.93 gm. discharge as the average weight of the second ejaculate; and in the case of the oldest animal here recorded, the weight was 1.75 gm.

Effects of prepuberal castration.—In order to study further the effects of testis hormone on the function of the prostate and seminal vesicles, as evidenced by the electrical ejaculation technique, a series of 15 young males were castrated and subjected to electrical stimulation. With the exception of 2 males, castrated at 5 and 8 days of age, all animals had been subjected to electrical stimulation at least once prior to castration.

Omitting details on individuals, the following results were obtained from this series of animals. (1) All males that had failed to give a coagulable discharge upon electrical stimulation pior to castration failed entirely to produce an ejaculate subsequent to castration; this was true despite stimulations given during several weeks after operation. (2) Animals castrated prior to day 40 failed entirely to deliver an ejaculate subsequent to operation. (3) Animals producing ejaculates prior to operation never again produced an ejaculate equal in amount to that delivered on the day on which castration was performed. (4) All animals failed to produce more than two coagulable ejaculates subsequent to castration, stimulations being administered each seventh day after operation.

Thus the average normal male produces a coagulable ejaculate by 36 days after birth, in an amount of 45 mg.; and the amount increases each week until maturity is established. The castrated animal either fails to produce ejaculates, in the event this function had not already been established before operation, or exhibits immediate marked regression in this function.

APPEARANCE OF SPERMATOZOA IN THE EJACULATE

The appearance of germ cells in the developing animal may be indicated from histological examination of testes, from smear preparations of an excised epididymis, or from age at first fertile matings. Since an ejaculation induced by the electrical method involves a reaction of all parts of the tract similar to that occurring under normal mating conditions, it was established as a routine to examine all discharges in saline solution in order to determine the age at which spermatozoa are discharged sufficiently mature to exhibit a normal type of motility. It is again to be emphasized that stimulations were given each seventh day after the initial stimulation. No attempt has been made to determine the age at which fertility is established.

Among the 55 young males whose ejaculation history was closely followed, a considerable degree of variability is found in the first discharge of spermatozoa. Fifty-four days was the earliest and 116 days the latest age at which first discharge of spermatozoa occurred; the latter animal had been subjected to fourteen consecutive weekly stimulations before spermatozoa were obtained in the discharge. More than one-half of the males voided spermatozoa by an age of 75 days, and only one reached an age greater than 100 days before spermatozoa capable of motility were obtained.

The data obtained from living healthy animals, and supplemented by a study of fixed materials (in subsequent sections), thus indicate the progression in acquiring normal reproductive functions. The average animal is capable of voiding its first coagulable seminal discharge on day 36. It becomes evident, therefore, that seminal vesicle and prostate function are acquired considerably earlier than the formation of spermatozoa, which occurred on day 54 at the earliest and on day 75 in one-half the males examined. Should one define "puberty" (as distinct from "maturity") as the time at which sperma-

tozoa capable of motility can be found in a typical coagulable seminal discharge, one could say that puberty is attained in 50 per cent of male guinea pigs by day 75 and in practically all males by an age of 115 days. The average animal at puberty will void a coagulable ejaculate of 300–400 mg. and will possess a body weight of 335 gm.

HISTOLOGICAL STUDY OF THE ORGANS OF THE REPRODUCTIVE SYSTEM

The foregoing account of the general growth-trends of various parts of the reproductive system and the study of the time of attainment of the various functions in the living animal have been closely correlated with a study of the histology of the various portions concerned. In this manner one is on somewhat more secure grounds in offering a physiological interpretation of structural conditions found in fixed preparations. The study of a close series of gonads and accessory organs should therefore be of some interest, especially since the tissues have been correlated with known physiological states determined on the living animal.

Testis.—Certain major features of interest in testis differentiation emerge from a close study of the organ from 77 males sacrificed at an age-range of from 2 to 130 days.

Prior to day 20 the testis consists of solid cords containing inactive spermatogonia and indifferent cells. A seminiferous tubule lumen appears in the majority of testes, from day 30 to day 40, and spermatocytes are present. Spermatids appear from day 45 to day 60, and metamorphosing spermatids ("sperm heads" of Moore, 1936) or spermatozoa were present in 1 case out of 13 between days 40–50, in 2 cases out of 10 between days 50–60, in 4 cases out of 5 between days 60–70; spermatozoa were present in the tubules almost invariably subsequent to day 70.

Spermatozoa are to be found in the epididymis shortly after sperm heads or spermatozoa appear in the testis. Although the earliest epididymal smear showing spermatozoa occurred between days 60 and 70, it will be recalled that in one case of electrical stimulation spermatozoa were present in the ejaculate on day 54: this specific animal was not sacrificed until day 62. Spermatozoa were usually present in the epididymis after day 70.

These observations, while showing a fairly rapid progression in the differentiation of germ cells in the testis, reveal that a greater discrepancy occurs in guinea pigs than in rats as to consistency in terms of age of spermatozoan differentiation (see Moore, 1936).

Seminal vesicles.—The seminal vesicles of 23 normal animals sacrificed at periods from 2 to 80 days of age were carefully studied histologically to determine in general the sequence of conditions from nonfunctional to the fully functional organ. This was supplemented by a study of the same organ from 67 animals castrated during this developmental period and sacrificed at varying periods thereafter. Thus, for example, 12 males were castrated between the fifth and eighth day of life and sacrificed periodically from day 15 to day 130. The total range of cases involved castrations at ages 1–73 days with sacrifices thereafter up to an age of $2\frac{1}{2}$ years.

In general appearance the cells of seminal vesicles of animals of 2–6 days of age are tall and columnar, tending in some limited areas to show a pseudo-stratified condition. The nuclei, in general, occupy a basal position; but the general cytoplasm fails to take a counterstain, excepting that the plasmalemma does stand out sharply. A transitional picture appears in animals 10–30 days of age. Many epithelial cells of the seminal vesicle have acquired a state that permits the cytoplasm to stain quite as does that of mature normal vesicles. Subsequent to day 30, the general appearance of the epithelium does not differ materially from the adult condition.

Castration at day 5 does inhibit the development of this general staining reaction of the epithelial cells, since sacrifice at day 45 or later reveals that the cells stain in accordance with the normal pattern of the 5-day-old pig.

Careful measurements of the epithelial height of seminal vesicles of all categories have been made by means of an ocular micrometer, to determine, first, the progression during normal development and, second, whether castration offers a modifying factor in development.

The results of the study of cell height have been somewhat surprising in the lack of any definite trend in effect, either in normal development or from castration. Variations in cell height determined from 23 normal males at ages of 2–80 days presented a range from 17 μ to 25 μ, or an average of 20 μ. Individual ranges have given no consistent increment in height, since in vesicles from animals 2 days of age cell height was greater than in vesicles removed from animals 60 days of age. The average cell height has therefore been attained by 2 days after birth.

Changes in cell height as a result of castration have been surprisingly inevident and inconsistent. One indication of involution in seminal vesicle epithelium of castrates occurs in cases where castration of short duration only begins at approximately the age of establishment of secretory function. Thus, an animal castrated at day 35 and sacrificed 10–20 days later may show an epithelial height of 11–13 μ, which is lower than any normal male in the series. On the other hand, another animal castrated on the same day but permitted to live for more than 2 years may show an epithelium appreciably higher. Thus, temporary reduction in cell height is suggested, but such reduction fails to characterize clearly a castrate condition.

Golgi apparatus.—The lack of any specific cell inclusion capable of definite diagnosis of the secretory state of the seminal vesicle of guinea pigs, such as portrayed by the large "secretion granules" of rat seminal vesicles by Moore, Hughes, and Gallagher (1930), suggested a study of the general responses of the Golgi body. The above-mentioned authors found definite castration modification in the Golgi bodies of the rat tissues.

The study of the progressive development of the Golgi body in seminal-vesicle epithelium of normal guinea pigs reveals that up to approximately 20 days of age the tall columnar cells contain a few osmicated granules either scattered or clumped in the lumen end of the cells. In slightly older males the cells reveal a more extensive granular state. The granules may be grouped in the lumen end of the cell, or occasionally the cytoplasm peripheral to the nucleus may be quite filled with osmophilic granules. By the forty-fifth day of life the previous copious supply of granules becomes aggregated into a knot approximately 8 μ in height and composed of a mass of coiled threads, usually running approximately parallel to the long axis of the cell. This condition characterized the cells of postpuberal and mature males.

Castration induces some modification in the differentiation of the Golgi body. In general it may be stated that the stage characteristic of normal males approaching maturity is not attained in animals castrated at an early age. Thus, castration prior to day 8 and sacrifice up to the forty-fifth day of age leave the Golgi apparatus in a juvenile state. The lumen end of the cell contains scattered osmicated granules or aggregates of granules. Animals castrated subsequent to the tenth day of age and permitted to live a year or longer still show reduced Golgi material in the seminal vesicle cells, but it is more typical of postpuberal castrates and may assume the form of a condensed nuclear cap. It becomes apparent that the histological condition of the seminal vesicles of the

guinea pig does not provide an adequate index of its functional state. Although electrical ejaculation proves that the vesicles have a normal function at an early age, there is a lack of clear histological condition to indicate it. There is an absence of cellular inclusions (well-defined granules) or of cell-height increase (or decrease after castration) that provides a reliable criterion for presence or absence of testis hormone.

Anterior prostate gland.—Studies on this portion of the prostate gland have been directed toward progressive differentiation with onset of its normal function and to effects that may be evident from castration; it is to be realized, therefore, that its structure in relation to function has been foremost in the course of these studies.

When cell height of the epithelial lining of anterior prostate acini is considered, one soon realizes that little difference exists between the cells of the inactive gland and those of one fully active. Careful measurements of cell height in glands removed at an age of 2–70 days indicated a range of 16–25 μ, or an average of 21 μ; greatest cell height occurred in the case of an animal 10 days old, and the lowest in a 15-day-old male.

Certain epithelial changes indicate progressions in development. The acinus lumen of the 2-day anterior prostate was 0.074 mm. in diameter, and this was progressively larger until in a 73-day-old prostate the lumen was 0.59 mm. The youngest anterior prostate epithelium is a simple columnar type. This gradually changes, with approaching maturity, into a pseudo-stratified condition, which changes further into a highly folded type. No characteristic cell inclusion of diagnostic value in determining the onset of active secretion was seen.

Castration at early stages exhibited itself principally by rather slight changes in the character of the epithelium and by an increase in size of the acini. Cell height appears to be unmodified by loss of testes. The epithelium tends to be decidedly less folded, and the cells are usually crowded together, producing a pseudo-stratified or stratified condition.

The osmic-acid technique for Golgi-body studies failed entirely in my hands. In anterior prostates of both young and mature males methods that proved successful with other tissues were unsatisfactory. On the whole, therefore, changes that denoted functional activity on the part of this gland failed to appear in either the progressive development or as a result of castration.

Middle prostate gland.—The middle lobe of the prostate gland does not differ markedly in its reactions from the anterior lobe. There is a progressive development from the columnar epithelial lining of simple acini to the folded epithelium of larger acini. Cell height cannot be closely correlated with age, at least from birth to puberty; and variations from 16 μ to 28 μ yield a general average of 22 μ.

Castration likewise does not markedly affect the histological character of this gland. There is some suggestion of a diminution in epithelial height when the testes are removed about the seventy-fifth day, but so many discrepancies enter that one is incapable of definitely diagnosing castration on the basis of general histological changes. Thus, the middle prostate from an animal castrated on day 35 and sacrificed more than 2 years later consisted of epithelial cells that fell within the range of cell heights in normal males.

Osmic acid impregnation of this portion of the prostate was fully successful despite the somewhat disconcerting results in attempting to relate specific morphological responses with a particular functional state. A definite Golgi body is present in the apical portion of middle lobe cells, and its general morphology in animals a few days old is

quite comparable to that of mature males. The body usually consists of a mass of coiled threads oriented in the long axis of the cell between nucleus and acinus end of the cell. Impregnation appears to be less intense in animals previous to 30 days of age than in mature males.

Golgi-body changes resulting from castration are more definite than is cell-height modifications. Castration between days 23 and 60, with sacrifice 100 days later, induces consistently a reduced Golgi body in the form of a cap against the nucleus of the cell. When operations were done from 2 to 8 days after birth, osmophilic material was present as delicate broken or continuous threads or as scattered granules. This suggests an inhibition of normal development in the Golgi body. The principal effects from castration, therefore, are reduction in size and in intensity of impregnation with osmic acid.

Posterior prostate.—A study of the posterior lobe of the prostate similar to that reported in slightly more detail for the anterior and middle lobes yields results approximating those obtained from a study of those portions of the gland. The lumina of the posterior-lobe acini are fairly characteristic in showing a progression from a diameter of 0.074 mm. in the case of a 2-day-old animal to 0.37 mm. diameter in a 60-day male. Cell height is again too variable to offer a diagnostic criterion of function.

Castration appears to induce a temporary reduction in cell height, but in cases of the longer periods of castration the cells often fall within the range of the normal males. The Golgi-body reactions of long-time castrates are again variable, but there is a decided tendency toward a diminished intensity in impregnation. In general the Golgi-body impregnation is sharpest in this lobe.

Vas deferens.—The vas deferens was studied in progressive sexual differentiation and in castration, but it likewise fails to offer outstanding structural conditions that can be definitely associated with a functional condition. The lumen of the tube increases progressively from 0.18 mm. prior to day 15 to a diameter of 0.40 mm. in 60-day males. The epithelial folds increase gradually in height; the cytoplasm of the cells stains deeply; and the apical ends of the cells are quite irregular.

Castration tends to reduce the diameter of the lumen, but considerable variability exists. No appreciable change in cell height follows removal of the testicle, and diagnosis of secretory states in this structure are difficult from general histological appearances.

Golgi-body reactions do not clearly indicate a secretory state in cells of the vas deferens as sexual differentiation progresses. The body lies in the long axis of the cell, apical to the nucleus. It is entirely similar in cells from the 6-day male and normal adult male. Castrations, particularly when performed at about 45 days of age, induce fairly distinct and consistent reductions in the osmicated materials, since the normal large knotlike mass becomes, in the castrated animal, sparse osmophilic granules scattered in the cytoplasm peripheral to the nucleus, or it may take the form of a thick threadlike structure. It is clearly reduced despite the lack of cell-height reduction.

DISCUSSION

The observations here reported were derived from a study of the progression in postnatal sexual differentiation in the male guinea pig. This study involved: (1) the individual growth of various parts, as exhibited by determinations of fresh weights on

carefully dissected organs; (2) the histological condition from birth to a well-developed functional state; (3) the study of the organs after castration performed at various ages; (4) the utilization of such functional tests as the capacity of spermatozoa to exhibit motility, the ability of seminal vesicles and prostate gland to provide normal secretions making up a coagulated ejaculate, and the ability of the animal to void such an ejaculate when properly stimulated.

Considering first the general growth of the reproductive parts, one is impressed with the sudden growth stimulus which occurs slightly earlier in the testis (at approximately the twentieth day) than in the accessory organs of reproduction (about 30–40 days of age). The curves of growth representing these trends fail, naturally, to portray individual variability; hence, analyses of individuals in respect to various aspects become enlightening. This analysis has been utilized both with respect to germ cell development and with respect to the internal secretory activity of the testes.

One of the most definite indexes of testis hormone secretion in the guinea pig is the voiding of a coagulable ejaculate, since this assures a functional state in both prostate and seminal vesicle, which function is under testis hormone control (see Moore and Gallagher, 1930). The observations reveal a fairly widespread variability in the attainment of this function in individual males. The youngest male to perform an ejaculation following electrical stimulation did so on day 21, whereas the oldest tested animal to first attain this capacity was 74 days of age. Fully one-half of the males delivered small coagulable ejaculates by day 30. This coincides approximately with the period of a rise in the curve of weights of the accessory organs. Thereafter both the growth curve and the curve of weights of ejaculate mounted upward until the early stage of maturity was reached. These responses therefore provide indications of the effectiveness of testis hormone.

The study of the progression in testis differentiation, carried on principally by histological study of the testes, was supplemented both by examining smear preparations of the epididymis and by an examination of the electrically induced seminal discharge for spermatozoa. Some variability again exists; but, on the whole, there is a fair consistency in the rate of germ-cell differentiation for all males studied. Metamorphosing spermatids (sperm heads) were first noted at an age of 48 days. This stage of development was attained by only 1 male among 13 examined between days 40 and 50; 3 among 10 males reached the sperm head or spermatozoan stage of development between days 50 and 60, while the majority of animals examined from 60 to 70 days had attained the same stage. One male only was found in which testes did not show spermatozoa at 70 days or later, and the age of this male was somewhat in question. On the whole, spermatozoa were not present in the epididymis until day 65, though one male voided a few motile spermatozoa in an ejaculation on day 54; motile cells appear in the ejaculate at approximately the same time as found in epididymal smears. In one case, only a few spermatozoa were found in the epididymis on day 73, when the ejaculate induced immediately prior to sacrifice failed to show them.

These considerations indicate a fairly rapid metamorphosis of germ cells, passage into the epididymis, acquisition of capacity for motility, and discharge through an ejaculation. Seventy days after birth represents a stage of development in the male guinea pig when the great majority of animals will void coagulable ejaculates containing spermatozoa that are actively motile.

The microscopical study of the accessory organs of reproduction yielded somewhat

surprising results in that structural conditions of the various parts change very little with the onset of function in normal progressive differentiation or with the loss of hormone following removal of the testes. A greater rapidity and extent of structural change with modifications in the hormonal state would be expected to be more apparent in this rodent in the light of such outstanding changes in the rat and ground squirrel (see Moore, Price, and Gallagher, 1930; Moore, Hughes, and Gallagher, 1930; Price, 1936; Wells, 1935). Whereas in normal differentiation the general character of the cytoplasm gives some indication of the changing functional conditions, cell height is modified but little. The height of the mature epithelium in many of these organs has already been attained at 2 days of age, and in early or later stages of castration there is only slight modification. Histological detail does not readily permit distinguishing the accessory reproductive organs of a male castrated longer than a year from those of normal males. In no technique employed by me have distinctive or diagnostic cell inclusions been found that were capable of denoting a definite functional state on the part of these accessory glands of reproduction. The condition of the Golgi bodies has most nearly approached this capacity. In certain portions (posterior prostate, and vas deferens) a clearly progressive differentiation was evident; the conditions are more or less characteristic of each tissue studied, and in some of these castration induced fairly evident changes from the normal. In other organs, however, progressive development failed to present apparent differences, and castration often produced such slight changes as to make it almost impossible to diagnose the state as one of lack of function lost through castration. The guinea pig, therefore, is a poor test animal for the study of effects of testis hormone on the histological state of the accessory reproductive organs. The electrical ejaculation test, on the other hand, and possibly also the spermatozoan motility test (not used by me; see Moore and McGee, 1928), is a useful test method.

In a later paper more detailed studies will be reported from the study of the effects of castration in mature animals.

SUMMARY AND CONCLUSIONS

1. Growth-rates of testes and accessory reproductive organs of male guinea pigs are accelerated from days 30–40; thereafter growth is rapid.

2. Ejaculations are induced as early as day 21, and the majority of males demonstrate this capacity by approximately day 30.

3. Spermatozoa first appear in the testis about day 50; they are fairly uniformly present by day 70. Spermatozoa appear in the epididymis and are discharged in an electrically stimulated ejaculate within a few days from their first appearance in the testes.

4. Histological study of accessory organs of reproduction during the age of functional development shows a lack of progression in cell-height increases. Prepuberal castration exerts but little modification in prostate and seminal vesicles. Some tendency for lowered cell height is suggested in castrations subsequent to about day 30. No specific cytoplasmic inclusions of diagnostic value for the process of secretion have been noted. Golgi-body changes provide some evidence of lack of testis hormone but are too variable to be of diagnostic value.

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MOTOR AND SENSORY HYPERPLASIA FOLLOWING LIMB-BUD TRANSPLANTATIONS IN CHICK EMBRYOS[1]

(Seven figures)

VIKTOR HAMBURGER
Washington University

THE factors controlling growth and differentiation of the vertebrate nervous system have been extensively studied in amphibian embryos. The experimental analysis revealed three main groups of factors: (1) patterns of local proliferation and differentiation, (2) mutual correlations between the different regions of the central nervous system, and (3) growth-controlling factors residing in the nonnervous peripheral structures, to be innervated (see reviews by Harrison, 1935, and Detwiler, 1936). The present report deals exclusively with the last-mentioned factors.

For *Amblystoma*, Detwiler and others have shown that spinal ganglia react sensitively to a decrease or an increase of their peripheral fields. A reduction of their peripheral area (e.g., by the extirpation of a limb) results in a hypoplasia of approximately 50 per cent, whereas peripheral overloading (e.g., by implanting an additional limb) causes a hyperplasia up to 40 per cent. In both instances the spinal cord and, in particular, its motor area showed no reaction; its cell number was in no way changed by the changes in its peripheral fields. Dürken (1912) and May (1930, 1933), on the other hand, reported positive reactions of the spinal cord of the frog, following the same type of operations: a hypoplasia of the motor column after limb-bud extirpation, and its hyperplasia after implantation of an accessory limb. Results similar to the last mentioned were obtained on chick embryos. A marked hypoplasia of the lateral motor column, in addition to hypoplasia of the corresponding spinal ganglia, was found when wing buds were extirpated in chick embryos incubated 3 days (Shorey, 1909; Hamburger, 1934). The difference in the reaction of the motor cells between *Amblystoma*, on the one hand, and the frog and chick, on the other, was attributed to the absence in the former forms, and the presence in the latter, of a distinct lateral motor horn (see Hamburger, 1934, p. 488).

The mechanism through which the peripheral structures affect processes going on in the central nervous system is still obscure. Considering, for a moment, the reaction of the motor horn in the chick, the only clue we have found is in the following observations: (1) the spinal ganglion and the motor column of the same segment react independently of each other; and (2) the motor hypoplasia is proportional to the amount of loss of muscle tissue. Obviously, we are dealing neither with a growth stimulus transmitted through a reflex arc nor with a mass reaction of the central nervous system, but with a localized, almost point-to-point, stimulation of a certain area of motor neuroblasts by their own end-organs (differentiating muscles). The tentative explanation was proposed that a growth-controlling agent travels in centripetal direction along the pathfinder motor fibers which first reach the peripheral area. This agent would be produced in a quantity proportionate to the size of the peripheral area to be innervated and would control, in a quantitative way, either the mitotic activity or certain steps in the differentiation of the neuroblasts which lie in the neighborhood of the pathfinder neurons (Hamburger, 1934).

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It was hoped to obtain more information on this point by studying the reaction of the motor horn of the chick to peripheral overloading. Its effect must not be necessarily the reverse of the hypoplastic effect following a reduction of the peripheral area. In the case of hypoplasia, one might still be inclined to attribute this growth inhibition to a general, nonspecific factor. On the other hand, a positive effect in the form of a localized growth stimulation of a given region of the motor column beyond its normal size, exerted by an enlargement of its own peripheral field, would strongly support our assumption of a specific growth-controlling mechanism.

THE OPERATION

Our experimental approach was the same as in the respective amphibian experiments; certain levels of the spinal cord were overloaded by implanting a supernumerary limb into their vicinity. The operations were made on chick embryos at 60–70 hours of incubation, i.e., at a time when the first motor fibers begin to emerge from the spinal cord. In these stages the wing and leg buds project as small buds from the flank of the embryo. The donor embryo was removed from the blastoderm, transferred into a watch glass containing 0.9 per cent salt solution, and the wing or leg bud excised with an iridectomy knife. The host embryo was then prepared: a window was sawed into the shell; a longitudinal slit was made with a glass needle into the flank near the outer borders of the somites; and the transplant was placed in this slit. It usually became attached to the body wall of the host embryo. The window was closed, and the host embryo was returned to the incubator for a period of 8–9 days. The technique has been described in detail in earlier papers (Hamburger, 1938, 1939).

THE INNERVATION OF THE TRANSPLANTS

Our expectation that host nerves would enter the transplants was fulfilled in many cases. However, it was found that only the transplants located near the spinal cord received an appreciable nerve supply; those located ventrally, or others which were attached to the mesenteries, either remained nerveless or were poorly innervated. The dorsal transplants were supplied by nerves originating in the adjacent levels of the spinal cord of the host, i.e., transplants located near the host wing were supplied by brachial nerves, transplants located near the host legs were supplied by lumbar nerves, whereas transplants located in the trunk region were entered by trunk nerves alone or by a combination of trunk and limb nerves. The number of nerves supplying a transplant was usually smaller than that of a normal wing or leg. Any nerve or nerves that entered a transplant formed a more or less complete and typical nerve pattern within the limb, irrespective of their source of origin and of their quantitative development. These and the following observations are based on reconstructions from serial sections of the innervation of thirty-two transplants (Hamburger, 1939).

THE QUANTITATIVE DEVELOPMENT OF TRANSPLANT NERVES

A comparison of transplant nerves with their partners on the left (unoperated) side showed that they were hyperplastic. However, their increase in diameter was always limited and never very conspicuous. As a result, the ingrowing nerves did not meet fully the increased demand, the innervation of a transplanted limb always remained far below saturation. In only a few cases did we find an increase in nerve diameter above 100 per cent. Since the number of transplant nerves was always smaller than normal, each one would have to enlarge by several hundred per cent if a quantitatively complete in-

nervation were to be accomplished. This growth limitation of the transplant nerves proved to be a serious impediment to the present study. If a considerable enlargement of the peripheral fields causes only a slight increase of the peripheral fibers, then no marked change in the number of neurons can be expected.

TABLE 1

TOTAL CELL COUNTS OF LATERAL MOTOR HORN CELLS

Position of Transplant	Case No.	Age (Days)	Transplant Innervated by Nerves Number	Sections Counted Every—	Totals	
					Total Number*	Difference
					Brachial Swelling	
Wing in wing-level	tre 419	9	15–17	other	r=7,217 l=6,684	533 (8%)
	tre 379	9	16	third	r=4,106 l=3,890	216 (5.5%)
Control	9n1	9	13–16	other	r=6,740 l=6,634	106 (1.5%)
					Lumbosacral Swelling	
Leg in leg-level	tre 21	8	21–23	third	r=5,969 l=5,120	849 (16.5%)
	tre 393	9	22–24	other	r=9,112 l=7,980	1,132 (14.2%)
	tre 347	8	22, 23	third	r=5,797 l=5,899	−102 (−1.7%)
	tre 330	8	22, 23	third	r=5,585 l=5,545	40 (0.7%)
	tre 340	8	21–24	third	r=6,246 l=6,261	−15 (−0.25%)
	tre 355	9	23, 24	third	r=6,149 l=6,206	−57 (−0.9%)
	tre 383	9	23, 24	third	r=5,695 l=5,671	24 (0.44%)
Control	9n1	9	23–29	other	r=8,271 l=8,238	33 (0.4%)

* Symbols: r=right; l=left.

For reasons discussed in the introduction we are particularly interested in the reactions of the motor centers. Unfortunately, motor fibers are more scarce in transplants than are sensory fibers. In several transplants numerous fibers were found to approach and to enter the skin, but only a few entered muscle fibers. In such cases the hyperplasia of the peripheral nerves must be due largely to an increase in sensory fibers. This fact adds to the above-mentioned difficulty in obtaining a demonstrable motor hyperplasia.

TABLE 2

SEGMENTAL CELL COUNTS OF LATERAL MOTOR HORN CELLS IN THE BRACHIAL REGION

Case No.	Thirteenth Segment		Fourteenth Segment		Fifteenth Segment		Sixteenth Segment	
	Total Number	Difference	Total Number	Difference	Total Number	Difference	Total Number	Difference
Tre 419	r=1,225 l=1,225	0 (0%)	r=2,544 l=2,508	36 (1.4%)	r=2,375 l=2,103	273 (13%)	r=1,073 l=848	225 (26.5%)
Tre 379	r=417 l=437	−20 (−4.6%)	r=1,306 l=1,341	−35 (−2.6%)	r=1,570 l=1,439	133 (10%)	r=813 l=673	140 (20.8%)
Control 9n1	r=392 l=430	−38 (−8.9%)	r=1,916 l=1,978	−62 (−3%)	r=2,685 l=2,639	46 (1.7%)	r=1,747 l=1,590	157 (10%)

TABLE 3

SEGMENTAL CELL COUNTS OF LATERAL MOTOR HORN CELLS IN THE LUMBOSACRAL REGION

Case No.	Twenty-third Segment		Twenty-fourth Segment		Twenty-fifth Segment		Twenty-sixth Segment	
	Total Number	Difference	Total Number	Difference	Total Number	Difference	Total Number	Difference
Tre 21	r=462 l=245	217 (88%)	r=922 l=765	167 (22%)	r=894 l=804	90 (11%)	r=900 l=660	240 (36%)
Tre 393	r=1,205 l=1,040	165 (15%)	r=1,918 l=1,196	722 (60%)	r=1,785 l=1,192	593 (50%)	r=1,240 l=1,429	−189 (−13%)
Control 9n1	r=957 l=948	9 (1%)	r=1,543 l=1,573	−30 (−1.9%)	r=1,280 l=1,244	36 (2.8%)	r=1,342 l=1,246	96 (7.7%)

Case No.	Twenty-seventh Segment		Twenty-eighth Segment		Twenty-ninth Segment		Thirtieth Segment	
	Total Number	Difference	Total Number	Difference	Total Number	Difference	Total Number	Difference
Tre 21	r=907 l=868	49 (5%)	r=947 l=847	100 (12%)	r=569 l=595	−36 (−4%)	r=368 l=336	32 (9%)
Tre 393	r=1,345 l=1,231	114 (9%)	r=779 l=895	−116 (−15%)	r=840 l=997	−137 (−16%)	.....	.....
Control 9n1	r=1,258 l=1,310	−52 (−4%)	r=1,143 l=1,173	−30 (−2.5%)	r=748 l=744	4 (0.5%)	.....	.....

OBSERVATIONS ON MOTOR HYPERPLASIA

The wing- and leg-level of the spinal cord of the chick show large ventrolateral swellings, the lateral motor horns or columns. They include the motor neurons of the limb nerves. Between the lateral motor horn and the floor of the central canal is located the mesial motor column. Its fibers supply the axial musculature; their cell bodies and nuclei are smaller than those of the lateral motor cells. The lateral motor columns are absent in the trunk level between the brachial and the lumbosacral swellings, i.e., in the level of the seventeenth to the twenty-second segment.

We first considered the feasability of inducing the formation of a lateral horn in the trunk-level by overloading trunk nerves. Transplants located favorably in the dorsal trunk region and supplied purely by trunk nerves were, indeed, obtained. However, their innervation was quantitatively very poor; no large lateral motor neurons were discovered in the respective level of the spinal cord, and not even the mesial motor group showed any appreciable hyperplasia.

It was therefore decided to place the transplant in a position where one of the limb plexuses would contribute one or more nerves to its innervation, so that any reaction would appear as a hyperplasia of an existing lateral motor column. This was done in two ways: a leg bud was transplanted immediately in front of the host leg bud, or a wing bud was transplanted immediately behind the host wing bud. The slits in the somatopleure of the host which were to receive the transplant were extended into the base of the respective host bud. We thus obtained leg transplants which were partly innervated by posterior trunk nerves and partly by anterior lumbosacral nerves 23 and 24 (or exclusively by the latter two), and wing transplants which were innervated exclusively or predominantly by the posterior brachial nerves (15 and 16). Nine such cases were selected for further studies. The innervation of these cases is indicated in Table 1.

Method of cell counting.—The establishment of a hyperplastic effect is based on the comparison of cell numbers of the right (overloaded) and the left side of the spinal cord. Cross-sections, 10 μ thick and stained with Heidenhain's hematoxylin, were used. The lateral motor neurons can be identified readily by their large size and the dark deep stain of their cell bodies. Their nuclei were counted on both sides on every other or every third section of the entire brachial or lumbosacral swelling. Only nuclei containing nucleoli were counted. The considerably smaller cells scattered in the motor horn, which are presumably glia cells and few in number, were omitted. The nuclei were counted with a Veeder counter and, at the same time, were checked on paper by use of a camera lucida, so that each cell would be counted but once.

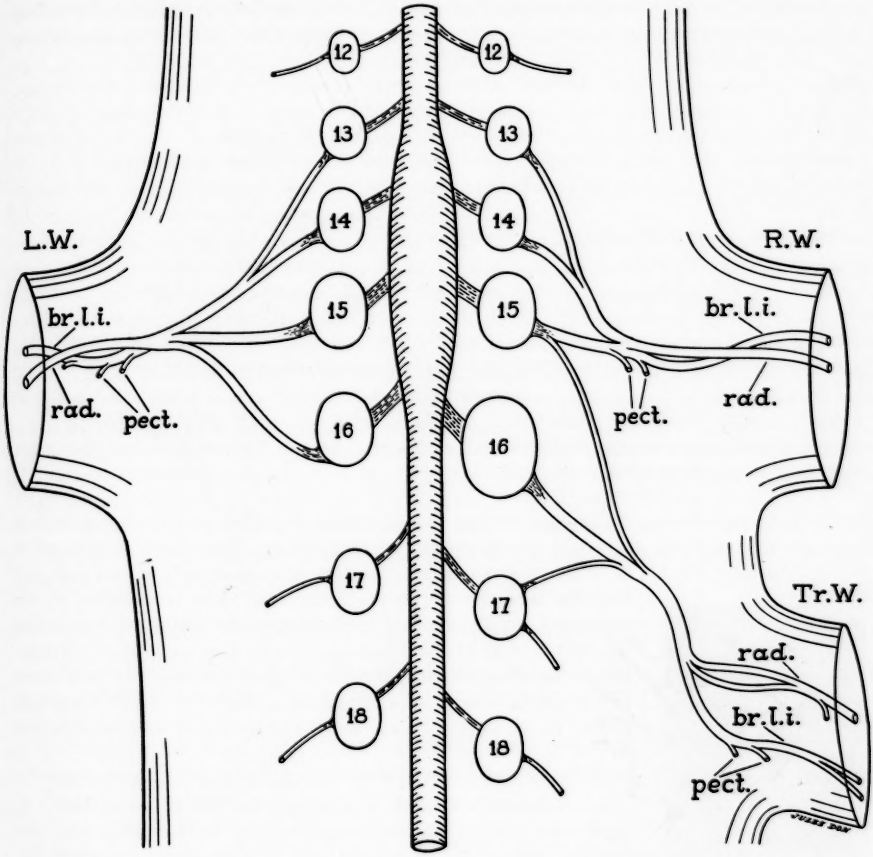
FIG. 1.—Tre 419. Right wing transplanted posterior to right host wing. Age at fixation, 9 days.

MOTOR HYPERPLASIA IN THE BRACHIAL SWELLING LEVEL

Two cases were selected in which a transplanted wing was located immediately posterior to, and in the same horizontal level as, the right host wing. Both transplants showed spontaneous motility.

Tre 419.—The transplant was developed normally but was slightly smaller in size than the host wing (Fig. 1). It was innervated (Fig. 2) by nerve 16 and small branches of

nerves 15 and 17. All three united in a plexus before entering the transplant. They formed a typical wing nerve pattern; its two main nerves—nervus radialis and nervus brachialis longus inferior—and their main branches were traceable. Strong nerves to the



Tre 419

FIG. 2.—Tre 419. Graphic reconstruction of the innervation of the transplant. *L.W.*, left host wing; *R.W.*, right host wing; *Tr.W.*, transplanted wing; *Br.l.i.*, nervus brachialis longus inferior; *Rad.*, nervus radialis; *Pect.*, nervus pectoralis.

pectoral muscles emerged from the nervus brachialis longus inferior of the transplant. Not less than seventeen different nerve bundles were found to enter the muscles of thigh and shank. The innervation of the host wing was accomplished by nerves 13–15.

The right nerve 16 was larger than the left one; the right ganglion 16 was greatly hyperplastic; and the right ganglion 15 was slightly hyperplastic. No appreciable increase in the diameter of peripheral nerve 15 was detected.

The lateral motor neurons were counted in every other section of the brachial swelling. A total difference of 533 cells was found, the hyperplasia of the right side amounting to

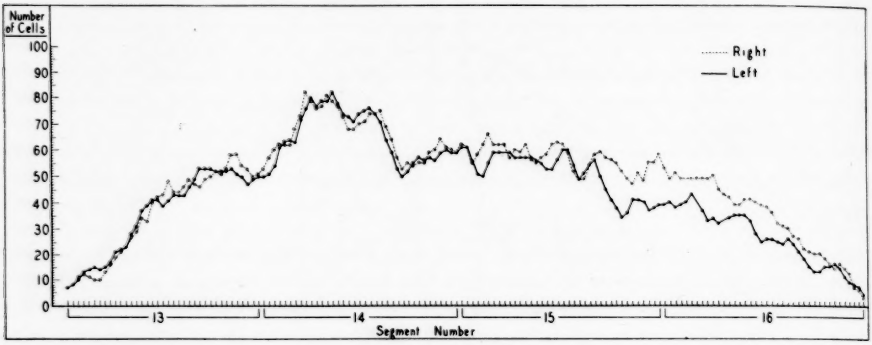


FIG. 3.—Tre 419. Comparison of the numbers of lateral motor neurons in the right and left half of the spinal cord (brachial swelling). Every other section was counted. Each point on the graph represents the average of five consecutive sections.

8 per cent. In order to obtain an accurate picture of the distribution of this hyperplasia over the extent of the brachial level, a graphic representation (Fig. 3) was constructed, in the following way: The ordinate is an estimate of the number of cells per section, and the abscissa the sequence of sections. The boundaries of the segments indicated on the abscissa were obtained by tracing the motor roots of the different nerves. The method of estimating the number of cells per section was to average the cell number in five consecutive counts, then omit the first count, add the next following count, and average these five counts, and so forth.

FIG. 4.—Tre 379. Right wing transplanted posterior to right host wing. Age at fixation, 9 days.

Table 2 shows clearly that no appreciable cell difference exists in segments 13 and 14 and that the hyperplasia begins in segment 15 and is highest in segment 16. In this latter segment the cell numbers of the right side exceed those of the left side almost with no exception. The percentages of hyperplasia, calculated for the four segments separately, are: 0 per cent in segment 13, 1.4 per cent in segment 14, 13 per cent in segment 15, and 26.5 per cent in segment 16 (see Table 1). Obviously, the hyperplasia is strictly localized in the posterior part of segment 15 and in segment 16. The latter supplies the transplant, while segment 15 contributes a branch to the transplant innervation and, in addition, may have to substitute for nerve 16 in the nerve supply of the host wing.

Tre 379.—The transplant (Fig. 4) is a fully developed wing immediately behind the host wing. Its innervation is supplied by nerve 16 (see reconstruction in Hamburger,

1939, Fig. 3). The strong transplant nerve branches into a nervus radialis and a nervus brachialis longus inferior and forms a typical, though incomplete, pattern in the transplant. The nerve bundles which were found to enter the transplant muscles are less numerous than in the preceding case. The nerves to the pectoral muscles of the transplant are completely missing. We may, therefore, expect a lower degree of hyperplasia than in tre 419. However, the right spinal ganglion 16 is considerably hyperplastic. The lateral motor neurons were counted in every third section. A total hyperplasia of the right horn, amounting to 5.5 per cent was established by the cell counts. This figure would not seem to be significant, although, when the cell numbers were plotted as before, a local hyperplasia in the sixteenth segment, amounting to 20.8 per cent, was detected. Again, the cell figures of the right side are consistently above those of the left side throughout the sixteenth segment.

A normal 9-day embryo was sectioned, and the motor neurons of its brachial region were counted on every other section. The total difference was 1.5 per cent in favor of the right side. The fluctuations within the four brachial segments were: $+8.9, -3, +1.7$, and $+10$ per cent, if the left side is taken as the basis. (This method makes the figures comparable to those of the operated cases.) The unaffected segments 13 and 14 of tre 419 and tre 379 can likewise be considered as control segments. We find here differences of $0, +1.4, -2.6$, and -4.6. per cent.

Compared with these fluctuations, those of segments 16 in the experimental cases, amounting to 26.5 and 20.8 per cent, respectively, must be considered as significant. The differences in segments 15 are perhaps doubtful. However, the graphs show that these slight increases are located at the posterior ends of the fifteenth segments and mark the beginning of the hyperplasia which is continuous through the sixteenth segment.

We conclude that the motor column of the brachial region can be stimulated to localized hyperplastic growth by peripheral overloading.

HYPERPLASIA IN THE LUMBOSACRAL SWELLING

The lumbosacral plexus of the chick (Fig. 5, left side) is formed by spinal nerves 23–29 (sometimes 23–30). Nerves 23, 24, and part of 25 unite in an anterior plexus, which subsequently forms the nervus cruralis. The posterior branch of nerve 25, together with nerves 26–29 (30), form a posterior plexus, from which the nervus ischiadicus emerges. Transplants located immediately in front of the host leg are usually supplied by a variable combination of posterior trunk nerves (21 and 22) and anterior lumbosacral nerves (23 and 24). Seven such cases whose nervous systems had been reconstructed previously were chosen for cell countings. Their innervation is given in Table 1.

A definite hyperplasia was found in two cases (tre 21 and tre 393); it amounted to 16.5 and 14.2 per cent, respectively. No hyperplasia was found in five cases. We shall take up the positive cases first.

Tre 21.—The transplant, a well-formed leg which showed spontaneous movements, was innervated (Fig. 5) by two trunk nerves, 21 and 22, and the anteriormost leg nerve, 23. Part of the latter, together with the former two, formed a joint plexus from which emerged the sciatic nerve of the host; part of nerve 23 entered the transplant directly and innervated its pelvic region. All these nerves were mixed, but their motor branches were not abundant. The right nerve 30 joins the lumbosacral plexus of the host leg. The total number of ganglia is 40 on either side.

Cell counts of the lateral motor nuclei of the entire lumbosacral swelling show a total difference of 849 cells (16.5 per cent).

In view of our observations on the brachial swelling we would expect to find this hyperplasia localized at the anterior end of the lumbosacral region and to find little, if any, difference in posterior regions.

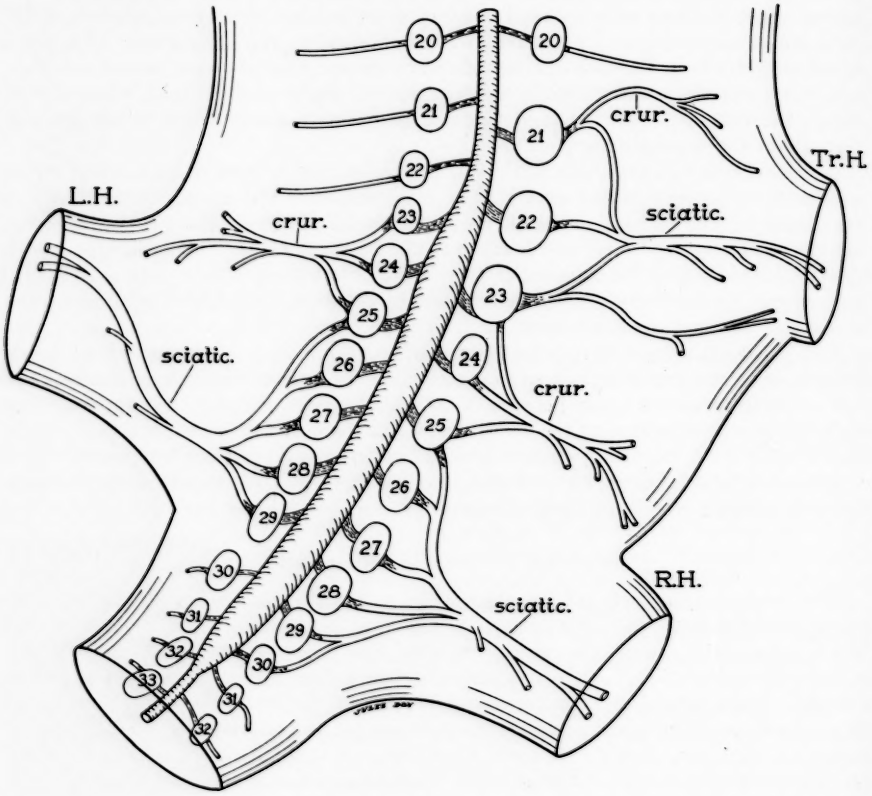


FIG. 5.—Tre 21. Graphic reconstruction of the innervation of the transplant. *L.H.* and *R.H.*, left and right host legs; *Tr.H.*, transplanted leg; *Crur.*, nervus cruralis; *Sciatic.*, nervus ischiadicus.

Unfortunately, the demonstration of a local effect in the lumbosacral level is made very difficult, for the following reason. When a transplant is located in front of the right host leg, the growth and expansion of its base causes a considerable bending of the posterior trunk and tail region of the host. As a result, the spinal cord is distorted, and no symmetrical cross-sections of it can be obtained in the critical level. This curvature of

the spinal cord is indicated in the shift of the spinal ganglia. As it is impossible to compare the left and right side section by section, another, less accurate, method was chosen. The motor roots of all spinal nerves involved were traced to the points of their emergence from the spinal cord. It was thus possible to establish with some approximation the boundaries between two consecutive segments on the left and the right side, separately, and to compare the cell numbers in corresponding left-half and right-half segments. Figure 6 presents the results in a graphical form; the actual figures are given in Table 3. The hyperplasia is most marked in the anterior segments 23 and 24 and in segment 26. The differences in the other segments are considered as doubtful.

The spread of the hyperplasia over other segments than the one which is actually involved in the innervation of the transplant can be interpreted as a compensatory effect;

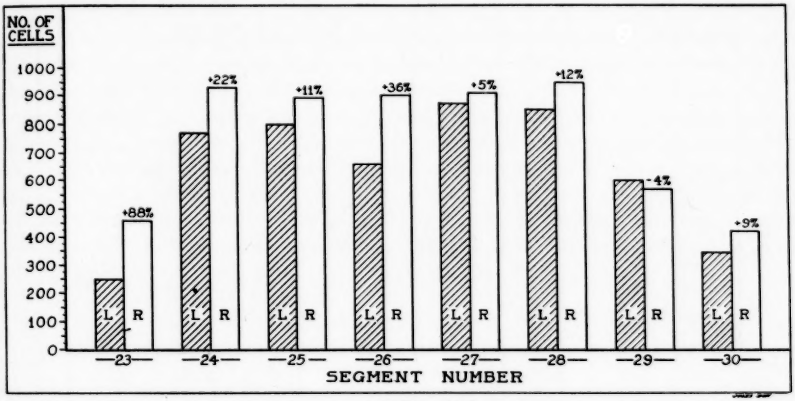


FIG. 6.—Tre 21. Comparison of the numbers of lateral motor neurons in the right and left half of the lumbosacral segments of the spinal cord. Every third section was counted.

an alternative explanation is that motor fibers originating in segments 24 and 26 grow craniad into the transplant nerves.

The trunk nerves 21 and 22 are considerably hyperplastic, as are their spinal ganglia. However, no hyperplasia was discovered in their motor centers. The number of mesial motor neurons was found to be equal on both sides (right side, 1,235 cells; left side, 1,238 cells). The hyperplasia of these nerves must therefore be accounted for either by an increase in sensory fibers only or, if the number of motor fibers is also increased, by splitting of peripheral fibers instead of by cellular hyperplasia. A third explanation would be that motor fibers originating in more caudal segments enter these transplant nerves. We favor the first assumption, as the total motor supply of the transplant is not abundant and might well be accounted for by the normal number of motor fibers present in nerves 21–22, increased by the considerable motor hyperplasia in nerve 23.

Tre 393.—The transplant, a normally differentiated leg (see Hamburger, 1939, Fig. 21) which showed spontaneous movements, was located anterior to the right host leg. It was innervated by trunk nerve 22 and by lumbosacral nerves 23 and 24. Nerve 24 also

contributed a posterior branch to the lumbosacral plexus of the host. Numerous motor fiber bundles were found to enter the transplant muscles.

The nuclei of the lateral motor horns were counted in every other section throughout the entire lumbosacral swelling. The total difference was found to be 14.2 per cent in favor of the operated side.

The trunk region of the host was again strongly bent; therefore, the same method as before was applied to calculate the percentage of hyperplasia per segment (Fig. 7 and

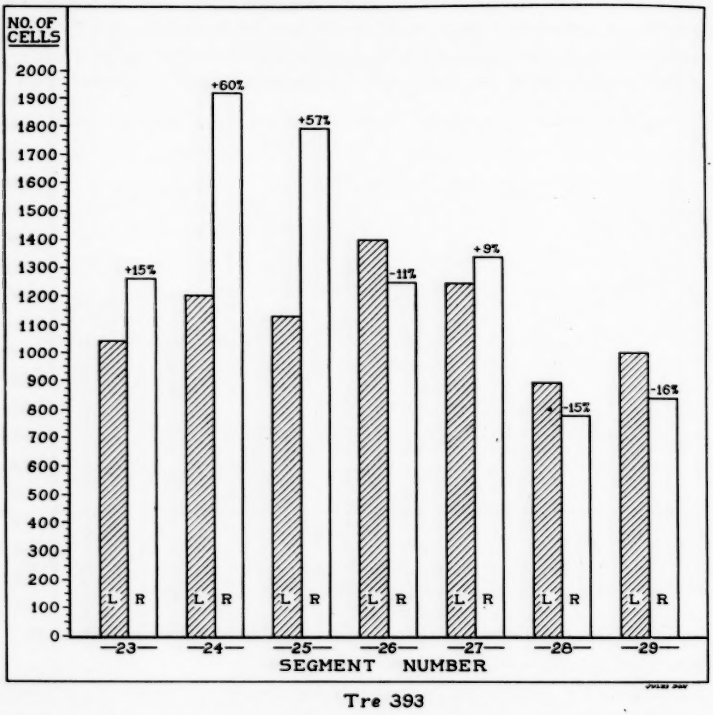


FIG. 7.—Tre 393. Comparison of the numbers of lateral motor neurons in the right and left half of the lumbosacral segments of the spinal cord. Every other section was counted.

Table 3). Again we found considerable differences in the anterior segments 24 and 25 (amounting to 60 and 50 per cent, respectively). The spreading of the hyperplasia over segment 25, which is not directly involved in the innervation of the transplant, may again be explained either as a compensatory effect (nerves 24 and 25 alone form the crurial nerve of the host) or by assuming that fibers from this segment grew craniad and entered transplant nerves. Nerve 23, apparently, does not contain an excessive amount of motor fibers.

Cell counts of the lumbosacral swelling of the control embryo 9n1 (on every other section) showed a total difference of only 33 cells (0.4 per cent). Since the embryo was

straight, an accurate comparison of left-half and right-half segments was possible. The differences within the individual segments ranged from 0.5 to 7.7 per cent. The differences in the unaffected posterior segments of the operated animals, which may also be considered as control segments, ranged from 4 to 16 per cent. It is obvious that the local differences of 50–88 per cent found in anterior segments of operated animals are significant.

Cell counts were made in five other cases of well-developed leg transplants. None of them showed any hyperplasia, the differences are below 1 per cent in four out of five cases, and in favor of the left (control) side in three cases. The absence of any reaction can be explained in two ways: either the motor innervation of these transplants was very poor, and no motor fibers in excess of those which are normally present in the transplant nerves were formed, or, if the motor supply was considerable, other mechanisms than cellular hyperplasia (e.g., a bifurcation of existing fibers) must have come into play.

Tre 347.—This case can be disposed of without further discussion. No motor hyperplasia was to be expected, as the only transplant nerve which originated in the lumbosacral level of the host (nerve 23) contained exclusively sensory fibers.

In the remaining four cases (tre 330, tre 340, tre 355, tre 388) motor fibers entering transplant muscles were exceedingly scarce; their number was considerably smaller than that of the four positive cases. It would seem that the amount of motor fibers normally present in nerves 23 and 24 would be quite sufficient to account for the motor innervation of these transplants, and no hyperplasia would be expected. Although the possibility of a peripheral bifurcation cannot be ruled out definitely, the cases under discussion certainly do not necessitate or even warrant such an assumption.

SENSORY HYPERPLASIA

It had been shown previously (Hamburger, 1934) that in the chick, as in amphibians (Detwiler, 1920), a decrease in the peripheral sensory area results in a marked hypoplasia of the corresponding ganglia. The present experiments show that an increase in peripheral area results invariably in a definite hyperplasia of the ganglia supplying the supernumerary limbs.

The following observations are based on a material of thirty-two sectioned and reconstructed cases.

In studying the peripheral distribution of the transplant nerves it was found that the sensory innervation, though by no means abundant, was more complete than the motor innervation. In accordance with this observation we found a hyperplasia in the spinal ganglion of almost every nerve which entered a transplant. Even slight increases in the size of transplant nerves were reflected in an enlargement of their ganglia.

Our method of reconstruction of the nervous system permits detection of even slight differences in the length of left and right ganglia. In order to obtain quantitative data, paper models of a selected number of ganglia were prepared and their weights compared, following a method applied by Detwiler (1920) and others. The complete sequence of sections of a given pair of left and right ganglia was outlined on cardboard with the camera lucida, cut out, and weighed. Data thus obtained give a rough estimate of the increase in volume (see Table 4). Every transplant ganglion shows an increase in volume with the exception of tre 419, ganglion 17. The branch from nerve 17 to this transplant is very small. The hyperplasia is highly variable, ranging from 15 per cent (which figure

may not be significant) to over 200 per cent. We suspect that this variation is quantitatively correlated with the variation in the increase of the peripheral fields, but no attempt was made to verify this point.

The table shows that brachial, lumbosacral, and trunk nerves react equally well. It brings out a further point of interest: the localization of the effect. With few exceptions only those ganglia responded which were actually involved in the supply of the transplant, whereas neighboring ganglia remained unaffected. Of the exceptions to this rule, some do not seem to be significant (e.g., tre 379, ganglia 14 and 15; tre 393, ganglion 21). The only case of a conspicuous hyperplasia of over 40 per cent in a ganglion not innervating a transplant is tre 383, ganglion 25. A plausible explanation of its excessive growth is that of a compensatory effect. This ganglion is the only one remaining of the three crural

TABLE 4

COMPARISON OF WEIGHTS OF PAPER MODELS OF SPINAL GANGLIA (IN GRAMS)

Transplant No.	Transplant Innervated by Spinal Nerves Number	Ganglion Number														
		Brachial				Trunk						Lumbosacral				
		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Tre 419	15–17	.....	.....	r=6 l=5.2	r=4.9 l=2.9	r=1.8 l=1.8	r=2 l=2	.....	.....	.....	.....	.....	.....	.....	.....	.....
Tre 379	16	r=1.2 l=1.2	r=2.5 l=2.2	r=3.2 l=2.8	r=2.6 l=1.9	r=1.1 l=1.1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Tre 510	13–16	r=1.9 l=1.3	r=4.7 l=3.6	r=5 l=4.4	r=3.9 l=3.1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Tre 346	21–23	.....	.....	.....	.....	.....	.....	r=1.1 l=0.9	r=1.1 l=1.1	r=1.4 l=0.9	r=1.9 l=1.1	r=1.7 l=1.5	.....	.....	.....	.....
Tre 383	23, 24	.....	.....	.....	.....	.....	.....	.....	.....	.....	r=1.5 l=1.1	r=3.2 l=1.2	r=2.4 l=2.1	r=1.7 l=1.2	r=3.7 l=3.5	.....
Tre 393	22–24	.....	.....	.....	.....	.....	.....	.....	.....	r=1.4 l=1.2	r=1.8 l=1.3	r=2.7 l=2.4	r=3.9 l=1.2	r=1.9 l=2.1	r=2.7 l=3	r=2.4 l=2.2

ganglia of the host, nerves 23 and 24 being involved in the innervation of the transplant. A hyperplasia is to be expected if ganglion 25 alone, as the reconstruction shows, is burdened with the formation of the crural nerve of the host. Several other cases can be easily explained in the same way.

We conclude that the reactions of the sensory centers, like those of the motor centers, are more or less strictly localized in the levels which are actually overloaded peripherally. We are, obviously, not dealing with a nonspecific mass action of the outgrowing transplant tissue on the spinal cord. Additional convincing evidence ruling out this alternative is given in the following case. Transplant tre 347 is innervated by nerves 20, 22, and 23, whereas nerve 21, incidentally, did not enter the transplant. Although all four ganglia developed at approximately the same distance from the outgrowing transplant tissue, only the ones which were actually overloaded showed a hyperplasia, whereas ganglion 21 remained unchanged. Another case presents the same feature.

DISCUSSION

An attempt was made to produce a hyperplastic effect in the lateral motor column of the brachial swelling of the spinal cord by implanting an additional wing near the host wing, and in the lumbosacral swelling by implanting a leg near the host leg. Cell counts demonstrated a hyperplasia in four out of nine cases (two in the brachial, two in the lumbosacral region). The percentages of hyperplasia, if calculated for the entire brachial or lumbosacral swellings, were low, ranging from 5.5 to 16.5 per cent. However, when the percentage hyperplasia was calculated for individual segments of the spinal cord, then the hyperplastic effect was found to be localized in few segments, where it ranged from 13 to 88 per cent (average for eight affected segments, 37 per cent), whereas other segments remained practically unaffected.

It is of importance to notice that the hyperplastic segments were those from which the overloaded transplant nerves emerged, whereas the unchanged segments were those which did not contribute to the transplant innervation. The most striking illustration of this point was found in the two cases of brachial hyperplasia, where a point-to-point comparison between the left and the right side of the spinal cord was possible (Fig. 3). Here we found that in the overloaded posterior segments the cell numbers of the right side were constantly above those of the left side, whereas they were practically identical, section by section, in the unaffected anterior segments. A spreading of the hyperplastic effect over segments which were not overloaded but were immediately adjacent to "transplant" segments was suggested as a compensatory effect.

This intimate correlation cannot be incidental. We consider these data as additional evidence for a direct growth stimulation of local motor centers in the spinal cord by the peripheral muscle areas which these centers innervate. As was pointed out in the beginning of the article, the simplest and most satisfactory explanation of the mechanism involved here would be to assume a growth-controlling agent traveling in centripetal direction from the periphery along the first motor fibers to the growing motor centers. Alternative explanations—e.g., the transmission of the stimulus via a reflex arc, or a general growth reaction of a certain level of the spinal cord to adjacent, rapidly growing transplant tissue—are not supported by the facts. The former possibility is made unlikely by the independent size variations of spinal ganglia and motor columns in the same segment; and a mass reaction is practically ruled out by the observation that only segments which actually innervate the transplant show hyperplastic effects, whereas neighboring segments which are not overloaded but which are close to the transplant do not, in general, show any effect. A corresponding situation was found in experiments on wing extirpation (Hamburger, 1934).

A similar correlation exists between the increase in peripheral sensory fields and the growth of the corresponding spinal ganglia.

The five negative cases do not invalidate this conclusion but find a satisfactory explanation in the observation that these transplants had received an extremely poor motor innervation or none at all.

Is the increase in cellular proliferation in the positive cases sufficient to account for the excess of motor fibers found in the transplant? Or is it necessary to assume that some of the additional motor fibers were produced by a peripheral splitting of axons? This mechanism of fiber increase has been described for amphibians (Weiss, 1931, 1937; Detwiler, 1933, p. 290). In the best-analyzed case (Weiss, 1937) it was found that if in old

larvae of *Amblystoma* one brachial nerve was cut and introduced into an implanted accessory limb, the subsequent hyperplasia of this transplant nerve was accomplished by a bifurcation of peripheral nerves and not by a cellular increase in the motor center.

No quantitative data are available in our material to compare the central, cellular hyperplasia with the peripheral, fibrillar increase, but it seems that they are approximately in agreement. The amount of accessory motor fibers present even in the best-innervated cases was small enough to be readily accounted for by the respective cellular hyperplasia. Our material does not, therefore, necessitate the assumption of peripheral bifurcation.

The surprisingly small amount of hyperplasia which is obviously related to the quantitatively poor nerve supply of the transplants deserves a brief comment. As was pointed out before (Hamburger, 1939), the pathfinder fibers succeeded in most cases to locate the transplant, but these first connections failed to receive an adequate reinforcement.

Why this failure? Wing-bud extirpations have resulted in a hypoplasia of up to 60 per cent of the total number of motor neurons. One is therefore inclined to attribute to a normal limb a considerable growth-stimulating activity. The failure of a transplanted limb to manifest such an activity at a corresponding order of magnitude may be due either to a central or to a peripheral inhibition. Either the potency of the spinal cord to produce an excessive number of motor neurons is limited or, if this potency is not restricted, the stimuli exerted by the transplant are weaker than those originating in a normal limb. The greater distance between the transplant and the spinal cord may play a role in many cases. The time factor must also be considered. The exploring pathfinder fibers may find the transplant in an advanced stage of differentiation, a fact which may make establishment of further connections and the transmission of centripetal stimuli more difficult.

COMPARISON WITH AMPHIBIA

Numerous experiments on Amphibia have shown that spinal ganglia and other sensory centers react sensitively by hyperplastic growth to an increase in the size of their peripheral sensory fields (review in Detwiler, 1936). In this respect the data on amphibians and on the chick are in full agreement. However, marked differences between different forms were found when the effects of peripheral changes on motor centers were tested. Detwiler (1924 and later) has given convincing evidence that the extirpation of one or even of both forelimb primordia, in *Amblystoma*, does not result in a decrease of the number of motor neurons in the corresponding segments of the spinal cord, the only perceptible effect being a slight size reduction of individual neurons (Detwiler and Lewis, 1925). On the other hand, Dürken (1912) and May (1930, 1933), using different genera of anurans, have described a motor hypoplasia following limb-bud extirpation. Similarly, wing-bud extirpations in 3-day chick embryos resulted in a marked hypoplasia of the lateral motor horn (Hamburger, 1934).

Corresponding discrepancies were discovered when the response of the motor center to peripheral overloading was studied. Detwiler summarizes his experiences with the urodele *Amblystoma* as follows: "When one, two or even three limbs are grafted to a heterotopic position and are innervated chiefly by the 6th and 7th nerves, the ventral motor areas of these nerves do not show any numerical increase in response to the added musculature" (1933, p. 290). On the other hand, May (1933), working on the anuran *Discoglossus*, described hyperplastic reaction of the spinal cord following the implanta-

tion of a hind-limb bud in a position near the normal hind limb. The present investigation again places the chick in line with anurans and in contrast to urodeles.

May's conclusions are based on cell counts in three of his five cases. He found an excess of cells on the operated side, amounting to 8–33 per cent in the segments innervating the transplant. His data are not directly comparable to my figures, as he counted all cells in the ventral half of the cross-section; and he counted only ten sections per segment. In one case, in which four segments adjacent to the "transplant" segments were counted as a control, three of these showed a hyperplasia of the right side of almost the same amount as the "transplant" segment. His data are altogether inadequate to permit any conclusion as to the actual amount and the localization of the hyperplasia.

The differences in the reaction of motor centers between anurans and the chick, on the one side, and urodeles, on the other, are obviously real, since they are demonstrated in experiments of overloading as well as reduction of peripheral fields. We have suggested that they might be due to structural differences in the spinal cord, the former types possessing a definite lateral motor column, which is missing in the more primitive cord of *Amblystoma* (Hamburger, 1934).

SUMMARY

1. Wing primordia of a 3-day chick embryo were implanted immediately posterior to the right wing bud of a host embryo of the same stage. They became innervated by posterior brachial nerves. Cell counts of the lateral motor neurons of the brachial region in two cases showed a motor hyperplasia on the operated side which was localized in the segments innervating the transplant and which amounted to 26.5 and 20.8 per cent, respectively, in the affected segments.

2. Hind-limb primordia were implanted immediately anterior to the leg buds of host embryos and received part of their innervation from the anterior lumbosacral nerves of the host. Cell counts of lateral motor neurons in the entire lumbosacral swelling showed, in two cases, a total hyperplasia of the right side amounting to 16.5 and 14.2 per cent, respectively. A precise determination of the localization is difficult for technical reasons; but the available data indicate that, again, the hyperplasia was localized in the segments which actually contributed to the transplant innervation. It amounted to between 20 and 88 per cent in the affected segments.

3. The spinal ganglia of transplant nerves showed a definite hyperplasia.

4. These data give further evidence that in the chick embryo the peripheral fields control the growth of both sensory and motor centers in the spinal cord.

POSTSCRIPT

In a recent publication, Yü-Ch'üan Tsang (*Jour. Comp. Neurol.*, **70** [1939], 1) showed that in a strain of unilaterally polydactylous mice the retrodorsolateral column of the motor horn was hyperplastic in twelve out of nineteen cases. This coincidence of a strictly localized motor hyperplasia and of a localized peripheral hyperplasia is in full agreement with the data reported above, and suggests that in mammals the same causal relation exists between peripheral growth and nervous growth as in the frog and the chick. Tsang considers the growth abnormality of the central nervous system as the primary cause of polydactyly. This interpretation is untenable. His own negative results in seven of nineteen cases demonstrate that hyperdactyly can occur in the absence of changes of the central nervous system. Furthermore, we have shown that limbs of frog and of chick embryos will develop normally in the complete absence of any innervation (see Hamburger, 1939). Finally, the present paper demonstrates directly that a motor hyperplasia can be produced by an enlargement of the peripheral area to be innervated.

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THE PRODUCTION OF DUPLICITAS CRUCIATA AND MULTIPLE HEADS BY REGENERATION IN EUPLANARIA TIGRINA

(One plate and five text figures)

ROBERT H. SILBER AND VIKTOR HAMBURGER
Washington University and Marine Biological Laboratory, Woods Hole

THESE experiments are based on a modification of an operation first reported by J. van Duyne in 1896. He produced a duplication in planaria resembling the duplicitas cruciata which has been described for other animals, such as the salamander (Spemann, 1919; Wessel, 1926; Wittmann, 1929), the frog (Penners and Schleip, 1928), the scorpion (Brauer, 1917), *Tubifex* (Penners, 1924), and *Chaetopterus*

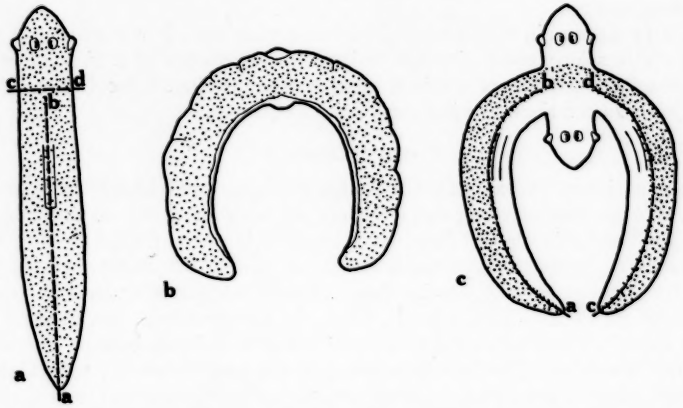

FIG. 1.—(*a*) Operation in Experiment 1; (*b*) 3 days after operation (note simultaneous appearance of anterior and posterior heads); (*c*) 8 days after operation (*a–b* and *c–d* indicate operation in Expt. 2). Clear areas indicate regenerated parts.

(Titlebaum, 1928). Duplicitas cruciata is characterized by two heads in opposite directions, separated by two tails also opposite each other and with their median plane perpendicular to that of the heads.

Van Duyne produced these duplications by making a single longitudinal cut through the median line of the planarian extending anteriorly to the head region. In a few cases in which the half-tails did not grow back together, a second head formed posteriorly at the anterior end of the slit. This operation has been repeated with occasional success by Morgan (1900), Bardeen (1901), Goetsch (1921, 1922), Keil (1924), and Beissenhirtz (1928).

We modified this operation in the following manner: a longitudinal cut was made through body and tail to a point halfway between the auricles and the pharynx (Fig. 1, *a* [*a–b*]), and then the head was removed by a transverse cut that left two half-tails

held together at their anterior ends by a narrow connection approximately one-third their width (*c–d*). The removal of the head permits the half-tails to assume a more outstretched condition and eliminates motion to a great extent. Both of these effects tend to produce a high percentage of successful cases. Beissenhirtz (1928) used the same modification. Duplications of the posterior heads were observed occasionally, but none occurred at the anterior cut surface.

In a second experiment the half-tails were allowed to regenerate for 8 days and were then split again in their new median planes. Here again supernumerary heads developed; but head development was distinctly lateral in most cases, in contrast to the crotch position found in the first experiment. The origin of such supernumerary heads was analyzed further in a third experiment.

Euplanaria tigrina (syn. *Planaria maculata*) was used in this experiment. The animals were collected from the pond behind the Episcopal church in Falmouth, Massachusetts (near Woods Hole), during August and early September, 1938. They were always used within 1 day after collection and were not fed. They were kept in tap water at room temperature.

Animals 11–14 mm. in length were placed on a glass plate in a few drops of water and were cut under a low-power binocular microscope by means of a piece of razor blade glued to the flattened end of a 5-inch piece of glass tubing. Narcotics were not used, since they have been shown to influence head frequency.

EXPERIMENT 1

In this experiment 100 animals (in groups of about 25 each) were cut, as shown in Figure 1, *a* (*a–b*). The pharynx was removed. After 24 hours the cut surface had healed over and the two half-tails had fused back together in varying degree. They were split with a needle to reopen the old longitudinal cut; and at the same time the head was removed by a transverse cut, leaving two half-tails held together by a narrow strip at their anterior cut ends (Fig. 1, *a* [*c–d*]). Thus, the transverse cut was delayed 24 hours.

About 10–15 minutes after the transverse cut, the animals became immobile in an outstretched condition. The two tails assumed a horseshoe shape, with their median longitudinal surfaces completely exposed and facing posteriorly and with their anterior surface only partially exposed, owing to extension of the half-tails and contraction of the edges. This combination of tail extension and contraction of the anterior cut surface brings the edges of the latter together and results in their partial fusion. The anterior cut surface thus becomes reduced in size (Fig. 2, *a–d*). In a few cases fusion was complete and no anterior surface was exposed (Fig. 2, *d*).

Since we found that the amount of anteriorly exposed tissue is correlated with the type of regeneration at the anterior end, we separated the animals 24 hours after the second cut into two groups: those with relatively much wound surface exposed anteriorly, and those with little or none.

Some individual cases were protocolled every day, but the majority were examined in groups of about 5–15 at least five times during the first 8 days after the operation. Final data were compiled on the eighth day after the transverse cut was made.

RESULTS

After the outstretched condition described previously had been assumed, the cut surfaces healed over smoothly—usually within the first day. Any minor irregularities on

the cut surface were completely smoothed over by the formation of new unpigmented tissue.

Distinct, unpigmented outgrowths appeared perpendicular to the anterior and posterior cut surfaces on the third or fourth day after the transverse cut had been made. Eyes became visible about 1 day later. Generally there was little or no visible difference in time of appearance of heads at the two surfaces (Fig. 1, *b*).

Regenerated pharynges were never observed earlier than the fourth day after the operation, and they did not fully differentiate for about 2–3 weeks. They originated close to the healed surface, that is, almost entirely in the regenerated tissue. Only after

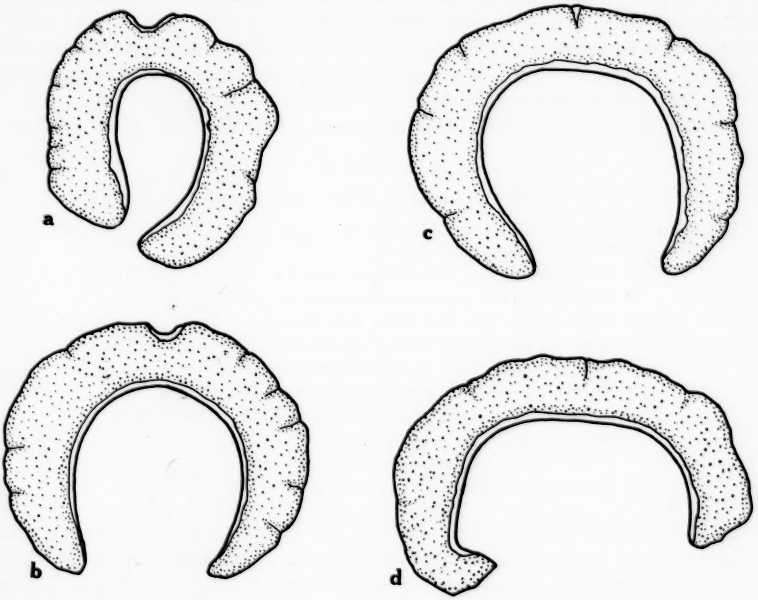

FIG. 2.—Variation in extent of fusion at the anterior cut surface 1 day after the transverse cut was made in Experiment 1.

lateral regeneration of the tail was complete, after several weeks, did the pharynges take up a median position in the tails. Double pharynges were found in several cases. Beissenhirtz (1928) also observed the lateral origin and duplication of pharynges.

Thus these processes may be said to occur more or less in the following sequence: healing, head formation, development of pharynges, and lateral regeneration of the tails.

After the heads differentiated, either head could take the lead, with the other head and two tails trailing behind. The heads alternated in guiding the movement, which was always either in an anterior or a posterior direction—never was the movement oblique or lateral.

Table 1 summarizes the results of Experiment 1. Anterior and posterior heads on the same animal were obtained in 79 per cent of the cases; anterior heads only, in 12 per

cent; and posterior heads only, in 9 per cent. At the anterior surface single heads developed in all cases (except the 9 cases in which no head formed), but duplicated heads formed posteriorly in 17 per cent of the cases. Abnormal heads were found anteriorly in 9 per cent, posteriorly in 21 per cent. Only 2 cases had both heads abnormal. These heads can be classified in Child's terminology (Child, 1915, p. 107). We found anophthalmic, teratomorphic, and teratophthalmic types.

Anterior heads absent (*Group 3 of Table 1; Fig. 9*).—The absence of anterior heads observed in 9 cases was definitely correlated with the amount of new tissue exposed anteriorly on the day after the transverse cut. An anterior head was entirely lacking when the edges of the anterior wound surface fused together so closely that, after healing, very little or no tissue was exposed (Fig. 2, *d*).

Anterior heads abnormal (*Group 1, d, e, and f*).—The 9 cases of this group, when observed on the day after the second cut, had less tissue exposed anteriorly than usual but more than the 9 cases mentioned in the preceding paragraph (Fig. 2, *c*). As expected, they developed abnormal anterior heads, either with two eyes, one eye, or no

TABLE 1

SUMMARY OF RESULTS OF EXPERIMENT 1

Total cases	..	..	100
1. Anterior and posterior heads	..	79	
a) Anterior normal, posterior normal	38		
b) Anterior normal, posterior abnormal	19		
c) Anterior normal, posterior duplicated	13		
d) Anterior abnormal, posterior normal	6		
e) Anterior abnormal, posterior abnormal	2		
f) Anterior abnormal, posterior duplicated	1		
2. Anterior heads normal, posterior heads lacking	..	12	
3. Anterior heads lacking, posterior heads present	..	9	
a) Posterior, single (and normal)	6		
b) Posterior, duplicated	3		

eyes, and no auricles in any case. At the time the data were taken (8 days), 5 of the 9 had no eyes, 3 had single eyes, and 1 had two eyes. (Some animals listed at this time as anophthalmic, however, were found to be teratomorphic, i.e., with single eyes, when a second inspection was made several weeks later.) It is obvious that incidental contraction and fusion is a major factor in determining the type of regenerate in these cases.

Anterior heads normal (*Groups 1, a, b, c, and 2*).—In 82 cases normal anterior heads developed. Of these, 77 had a considerable amount of tissue exposed on the day after the transverse cut (Fig. 2, *a* and *b*); the remaining 5 cases were doubtful when protocolled.

Posterior heads absent (*Group 2*).—Eight of the 12 cases of this category showed a partial fusion of the tails along the longitudinal cut surfaces. The other 4 were doubtful in this respect. Incidentally, all 12 cases belonged to that group which had a large amount of anterior surface exposed on the day after the second cut. It seems that their tails were less outstretched and more nearly parallel than usual (Fig. 2, *a*). This condition, of course, was more favorable for longitudinal fusion (or was caused by fusion), and at the same time prevented the usual decrease in anterior wound surface, owing to the bending and contracting that accompanies the assumption of the horseshoe shape. The

coincidence of large anterior surface and the absence of posterior heads can thus be explained on a simple mechanical basis.

Posterior heads abnormal (*Group 1, b and e*).—The group of 19 animals (Group 1, *b*) which developed normal anterior and abnormal posterior heads had much anterior surface exposed like Group 2. Thirteen of these had single posterior outgrowths without eyes or auricles—anophthalmic heads. In a few cases eyes were found imbedded in the tissue at the base of the outgrowth when whole mounts were prepared after several weeks. Either the cases diagnosed as anophthalmic were actually teratomorphic or the imbedded eye appeared later. The latter possibility is more likely—at least for a majority of these animals. The other 6 cases were teratomorphic heads.

Individual cases of these types were seen to develop the posterior protuberance at the same time as the anterior outgrowth, but then the anterior continued to develop a normal head while the posterior was inhibited.

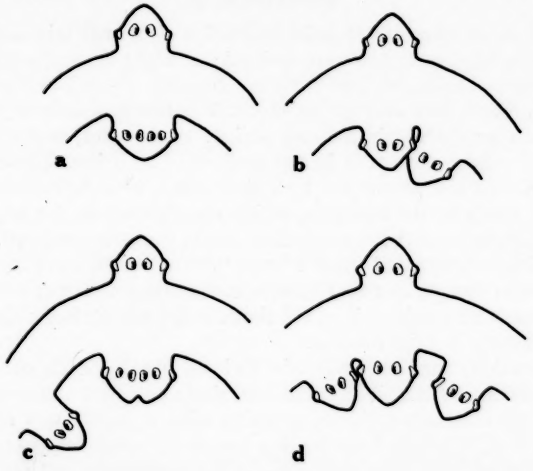

FIG. 3.—Types of posterior duplications found in Experiment 1

Only 2 cases (Group 1, *e*) were found in which both anterior and posterior heads were abnormal. The anterior heads were anophthalmic, and the posterior were teratophthalmic.

Posterior heads normal or duplicated (*Groups 1, a, c, d, f, and 3, a, b; Figs. 6–10*).—The interesting feature of this category was the appearance of duplications in 17 of the 67 cases in the group. Two or three distinct heads were found in 10 cases. The remaining 7 cases of duplication were more or less abnormal single or double outgrowths with supernumerary eyes. One of the most extreme cases had nine distinct eyes in two outgrowths.

The duplications were almost invariably crowded toward the crotch, which was always occupied by at least one outgrowth. One exceptional case is shown in Figures 3, *c*, and 10, in which one of two outgrowths was distinctly lateral and far removed from the

second, which was in the crotch as usual. The same specimen incidentally had four pharynges.

Following is a complete list of the duplications:

7 cases with 1 outgrowth and 3 or 4 eyes (Fig. 3, *a*)
3 cases with 2 outgrowths and 3 or 4 eyes (Fig. 3, *b*)
4 cases with 2 outgrowths and 5–9 eyes (Fig. 3, *c*)
3 cases with 3 outgrowths and 5–6 eyes (Fig. 3, *d*)

Beissenhirtz (1928) and Morgan (1900) obtained occasional cases of duplications.

Concerning the symmetry of the duplications, we found that in the 10 cases of head duplication one head was always in the crotch and the other or others were lateral to it. The 7 cases of partial head duplications with supernumerary eyes were always in the same plane of symmetry as the anterior head.

EXPERIMENT 2

In an attempt to compare crotch head formation of normal tails and of regenerating tails 20 cases of duplicitas cruciata were split again. Eight days after the transverse cut of the previous operation, a cut was extended through the tail and trunk to the head region (Fig. 1, *c*). The heads were not removed. The four half-tails were then kept apart by splitting with a needle within a 2-day period. Some cases, nevertheless, healed together again, and others were torn in the process of splitting. These were discarded. (The same experiment was attempted by Beissenhirtz, with little success.)

The cuts were made in the mid-line, which was located at the edge of the heavily pigmented or old tissue; and the regenerating pharynges were removed. Thus, two half-tails, for convenience designated "tail 1" and "tail 4," were composed entirely of old tissue; and the other two, tails 2 and 3, were composed almost entirely of regenerated, unpigmented tissue with a thin strip of old tissue at the cut surface. The old main nerve cords were presumably in tails 1 and 4.

After the operation, the four half-tails twisted around each other violently but eventually assumed an outstretched condition, similar to that assumed by the cases of experiment one. As mentioned above, splitting with a needle was necessary in most cases to keep the four half-tails from healing together; but seldom were they so treated more than once, never more than twice. Since the heads were not removed, these animals were kept in the dark, to eliminate as much motion as possible.

Head outgrowths appeared about 3 days after splitting (Fig. 1, *b*). The half-tails regenerated laterally, and small pharynges became visible in the regenerated tissue soon after the heads appeared, but never earlier than the fourth day.

The most striking features of these animals, as observed within 9 days after the operation, were the following:

1. Contrary to expectation, a large majority of the cases had no crotch heads but developed lateral heads on tails 1 and 4 (Figs. 11 and 12). Thus, of the thirty-one heads that developed, twenty-four (77 per cent) grew out laterally from tails 1 and 4 and seven (23 per cent) developed in the crotch or at least so close to it that they could not be readily distinguished from true crotch heads. Only those that were clearly not in the crotch were classified as "lateral." One case developed two new crotch heads, and thus became a radially symmetrical monster with four heads and four tails.

2. There were no lateral heads on tails 2 and 3.

3. The lateral heads, with the tails from which they developed, tore off from the rest of the animal after they had differentiated.

4. In 3 cases head duplication was observed—i.e., 1 case developed two lateral heads on tail 1, and 2 cases developed both a crotch head and a lateral head on the same cut surface. Five other cases had lateral heads with supernumerary eyes.

In connection with these duplications two specimens obtained in preliminary experiments at Cold Spring Harbor are of special interest. The species was not verified but was probably *Tigrina*. Both were cases of duplicitas cruciata that were split after 8 days like the animals of Experiment 2 (Fig. 1, *c*).

Case A (Fig. 4) was remarkable. Tails 1 and 4 developed lateral heads, and tails 2 and 3 did not, as in Experiment 2. However, tail 4 developed three lateral heads with two eyes each. Two of the three heads were fused. Tail 1 developed a broad lateral outgrowth along its regenerating surface, with six distinct eyes.

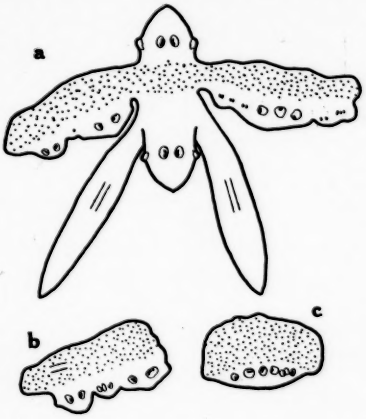

FIG. 4.—(*a*) Experiment 2: Cold Spring Harbor, case A, 6 days after operation as shown in Figure 1, *c*, lateral outgrowths on tails 1 and 4; (*b*) tail 4 after fission, 12 days after operation; (*c*) tail 1 after fission, 8 days after operation.

Case B developed only a single lateral head on tail 4; but tail 1, which was removed as a half-tail at the time of operation, developed nine distinct eyes in one broad lateral head outgrowth. Again tails 2 and 3 remained headless.

All four tails 1 and 4 described above tore off within 9 days, and three of them developed single pharynges no matter how many eyes or heads were present. The fourth, tail 1 of Case A, was lost.

The results of Experiment 2 are in contrast to those of Experiment 1, where there were relatively few lateral heads and a high percentage of crotch heads and where lateral heads were found only in addition to crotch heads. The difference might be due to the fact that the tails of the second experiment were partly composed of newly regenerated tissue when cut.

EXPERIMENT 3

This consideration led to the third experiment. If the tails of the duplicitas cruciata were allowed to differentiate longer, then they should more closely resemble the tails of an unoperated animal. Thus, 12 animals were allowed to differentiate for 16 days and were then cut in the same way as in Experiment 2. By this time the lateral regeneration was almost complete and the pharynges had taken up a position nearer the median line. Pigment had appeared in the regenerated tail tissue, although it was still not as heavy as in the older half of the tail. The behavior and development of these animals after operation was similar to that of the animals in Experiment 2. The results are summarized in Table 2.

TABLE 2

POSITION OF REGENERATED HEADS IN EXPERIMENTS 2 AND 3

	No. of Animals*	Total No. of Regenerated Heads	Lateral Heads on Tails 1 and 4	Lateral Heads on Tails 2 and 3	Crotch Heads
Expt. 2	17	31	24	0	7
Expt. 3	12	20	4	1	15

* In 6 cases two of the four tail parts fused back together.

As expected, the ratio of lateral heads to crotch heads was reduced, from 24:7 (in Expt. 2) to 5:15 (in Expt. 3), and approached the results of Experiment 1. The only essential difference was the occurrence in 4 cases of lateral heads in the absence of crotch heads, which was never observed in Experiment 1. We suspect that if differentiation had proceeded farther—that is, if the second longitudinal cut had been delayed more than 16 days—crotch heads would have developed even in these specimens.

One specimen requires special note. It had one lateral head on tail 1 and one on tail 3. This was the only case in which tail 2 or 3 developed a lateral head.

DISCUSSION

THE INTERPRETATION OF THE DUPLICATIONS AS DUPLICITAS CRUCIATA

"Duplicitas cruciata" is defined as a complete duplication in which the two heads, facing in opposite directions, have a common median plane of symmetry, and where the two tails likewise are opposite each other, with their median plane perpendicular to that of the heads. Such duplications have been described for *Tubifex* (Penners, 1924), *Chaetopterus* (Titlebaum, 1928), *Euscorpius* (Brauer, 1917), *Triton* (Spemann, 1919; Wessel, 1926; Wittmann, 1929), and *Rana* (Penners and Schleip, 1928). Some duplications obtained by transplantation in planaria (Miller, 1938) resemble duplicitas cruciata.

Those animals in our experiment which have one anterior and one posterior head fit the foregoing definition. In all these cases the posterior head was directly opposite the anterior. This position of the heads directly opposite each other was visible from the time of the earliest appearance of the head outgrowths (about 3 days).

The position of the nervous system was established by Bessenhirtz (1928). He showed that the nerve cords in the regenerated tissue are connected with the posterior head and that the anterior head is connected with the two old nerve cords. The position of the

four main nerve cords is indicated by dotted lines in Figure 5, *b*. The anterior parts of the intestinal trunks are continuous in the region between the heads and are bilaterally symmetrical (Fig. 8).

Observations of the movements of the animals after their heads had differentiated support our interpretation. Either head could take the lead, with the other head and tails trailing behind. In the same specimen neither head showed continual "dominance," but the heads alternated in directing the movements. No case was observed in which oblique or lateral movement occurred. The animals invariably moved either in an anterior or a posterior direction. All these observations confirm the interpretation that the duplication is a duplicitas cruciata.

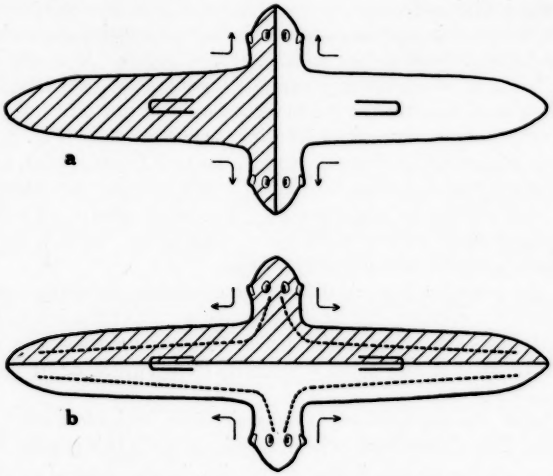

FIG. 5.—Diagrams indicating two types of duplicitas cruciata: (*a*) Spemann type; (*b*) Schultze type The dotted lines in *b* indicate the position of the main nerve cords.

According to Keil (1924), the posterior head would be the product of regeneration of one tail, and the anterior head would form a functional and structural unit with the other tail. Our observations rule out this interpretation.

Although all cases of duplicitas cruciata in the different animal groups have close geometrical resemblance, an analysis of their origin has made it necessary to distinguish between two different types.

The duplicitas cruciata represents a peculiar union of two individuals, in which either the heads or the posterior parts are of a composite nature—i.e., they are shared by both individuals. Schleip (1929) calls the forms with composite ("secondary") heads and uniform ("primary") tails the "Spemann type" (Fig. 5, *a*). The forms with "primary" heads and "secondary" tails (Fig. 5, *b*) he calls the "Schultze type," each type named after the investigator who first produced it experimentally in amphibians.

The two types differ in their embryonic origin. In the Spemann type, differentiation starts from two separate posterior centers instead of one. Differentiation proceeds in both along the same axis. The anterior ends of the primordial individuals meet and

deflect each other in such a way that half of each head primordium now differentiates perpendicularly to the original axis (see arrows in Fig. 5, *a*) and unites with the adjacent half-head of the other individual. The duplicitas cruciata that Spemann (1919) produced by fusing half-gastrulae in *Triton* and the duplicitas cruciata in *Tubifex* (Penners, 1924) and *Chaetopterus* (Titlebaum, 1928) belong to this type.

In the Schultze type, differentiation starts either from two separate anterior (scorpion, Brauer, 1917; arrows in Fig. 5, *b*) or from middle trunk centers (*Rana*, Penners and Schleip, 1928) and proceeds in either case to produce undisturbed primary heads and deflected secondary tails.

The present case is unique in that it does not have embryonic origin but results from regeneration of one individual.

To which type should this case be compared? In regenerating planarians a secondary head would have to be defined as one which has two sources; it would be composed of two "half-heads," each of which is the product of regeneration of one half-tail. If it were technically possible to vital-stain the regenerating cells of one half-tail, the regenerated head would be composed of one stained and one unstained half (Fig. 5, *a*). This interpretation has been advanced by Morgan (1900), Goetsch (1921, 1922), and Beissenhirtz (1928). The following considerations lead us to a different interpretation.

The anterior head cannot be considered a "secondary head." It originated from a single cut surface and never showed evidence of duplication. In fact, the end result can be obtained without removal of the anterior head.

The nature of the posterior head is difficult to establish. In every case of single posterior heads (71 of the 100 cases) the head appeared at the cephalic end of the posterior cut surface directly opposite the anterior head. The regeneration bud was single, i.e., showed no indication that it may have originated from two separate centers lateral to the plane of symmetry.

On the other hand, the duplications (17 cases) might indicate a composite nature of the posterior heads. The significance of head duplications in this connection is weakened by the fact that one head is usually in the crotch (i.e., in the plane of symmetry) and the other (or others) are more laterally located in an asymmetrical arrangement. We would certainly favor the secondary nature of the posterior heads if all the duplications were symmetrical with reference to the head axis. This, however, is rarely the case. In general the evidence favors the primary nature of the posterior heads—an origin from a single source rather than from two separate sources.

If one wishes to complete the comparison of this duplicitas with the Schultze type, one would have to consider the trunks and tails as secondary. This interpretation is favored by the relationship of the main nerve cords to the heads: the two cords of each tail are connected with two different heads.

Observations on contraction in the living animal several weeks after the operation demonstrate the same point. When contractions occur in the two tails and trunks, they take place simultaneously and in the same degree in both. Never is one tail contracted while the other is extended. This is true when either head is leading.

To summarize, this duplicitas cruciata resembles the Schultze type with primary heads and secondary tails.

This interpretation is in contrast to the interpretations offered by previous authors. Goetsch (1921, 1922) and Beissenhirtz (1928) consider the anterior head, as well as the posterior head, as secondary, each half of the posterior head regenerating from one half-

tail (i.e., the Spemann type, Fig. 5, *a;* see also p. 293). Their interpretation is based on the concept that the splitting produces two half-individuals which tend to reorganize by lateral regeneration like a planarian completely split through the median line.

This concept requires the rather strange implication that the anterior head, be it intact or regenerated, becomes "physiologically split" as a result of the individuation tendencies of the trunks and tails, so that eventually one half of the one head and the corresponding half of the other, together with the trunk and tail between them, constitute a new individual.

This assumption of "physiological splitting" under the influence of individuation tendencies set up by the posterior halves is contrary to Child's well-founded concept that a head dominates and thus individuates the parts posterior to it, and not vice versa. Our reasons for considering the posterior head primary have already been discussed.

Thus the interpretation of this duplicitas cruciata as the Schultze type with primary heads and secondary tails fits our observations best.

"BIPOLARITY" IN DUPLICITAS CRUCIATA

Bipolar heads have been frequently described in planaria (Morgan, 1898; Rustia, 1925; Watanabe, 1935; Miller, 1937; and others). They are usually produced from very short transverse pieces. The highest frequencies are obtained by operating at post-pharyngeal levels and by the application of a chemical agent, such as chloretone or strychnine.

In our experiment the two heads are certainly bipolar in the true sense of the word; their anteroposterior axes are in the same line but in opposite directions. However, the conditions which result in bipolarity are different in the two types of experiments. In transverse pieces the posterior head arises from a transverse cut surface, which would ordinarily be expected to regenerate a tail. This implies reversal of polarity near the posterior end, which may be a reversal of polarity in the ultra-structure or, more likely, a reversal of an activity gradient.

In the case of duplicitas cruciata no such reversal takes place. Although the point of origin of the posterior head is "posterior" to the anterior head and in this sense might be expected to form a tail, it is, at the same time, the most cephalic point of the longitudinal cut surface. This fact and possibly other local factors (to be discussed below) obviously provide such highly favorable conditions for head formation in the crotch that a head develops there in spite of the proximity of anterior tissue. Once head formation is determined at this point, the regeneration bud will grow out in a direction perpendicular to the cut surface. This is, incidentally, opposite in position and direction to the anterior head. In other words, this bipolarity does not make it necessary to assume a reversal of existing polarity.

In this respect we agree with Morgan, Goetsch, and others, who likewise did not consider the posterior head as a polar heteromorphosis.

FACTORS IN THE DETERMINATION AND LOCALIZATION OF HEADS

The previous discussion indicates our point of view that the problem presented by the duplicitas cruciata is not one of reversal of polarity or a problem of regulation on the part of two half-individuals connected at their anterior ends, but is a problem of head determination.

What factors are responsible for the origin and types of heads found in our experiments?

We shall discuss the following factors: (1) condition of the wound surface, (2) correlations between the two wound surfaces, (3) gradient systems, (4) nervous system, and (5) local factors determining lateral heads.

1. It has been pointed out previously (p. 288) that the incidence and type of anterior heads depends on the *amount of new tissue exposed* after the healing process. When the half-tails contract in such a way that the anterior surface can fuse completely, no head forms. The subnormal heads (anophthalmic, teratomorphic, etc.) are definitely correlated with partial fusion, which reduces the amount of newly healed tissue (Fig. 2, *c*). Subnormal or absent posterior heads can be explained similarly as a result of fusion at the crotch (p. 288). Thus, it seems that in our cases the total or partial inhibition of head formation can be adequately explained by the absence or paucity of wound surface.

Therefore, we would expect that, if fusion is complete at one cut surface, the other would be maximally outstretched and thus completely exposed. Indeed, whenever no head developed at the anterior surface, a normal head developed in the crotch, and vice versa (Table 1, Groups 2 and 3).

The importance of the wound surface in the formation of heads in cases of this type is further borne out by the results of a simple modification of Experiment 1. Completely headless, two-tailed animals can be produced in a high percentage of cases by delaying the transverse cut for at least a week. During this time the longitudinal cut surface has healed and partly regenerated. The anterior cut surface fuses completely, as in Group 3 of Experiment 1.

2. Does the development of either head exert *inhibitory or stimulating effects* on the other? Table 1 shows that this is not the case. In 50 per cent of all cases in Experiment 1, at least one normal head was present at each surface. The various combinations of normal, subnormal, and missing heads found in the other 50 per cent do not suggest any significant correlations. This is further borne out by the fact that a crotch head can be produced even when the anterior head is not removed. The occurrence of duplications at the longitudinal cut surface cannot be due to stimulation by the anterior head because 3 cases have developed in the absence of an anterior head and 1 case in the presence of a subnormal anterior head.

In this connection, a preliminary experiment with 4 animals was made in which the anterior surface was increased by a modification of the transverse cut in the first experiment. Instead of a single cut made directly through the animal, two oblique cuts were made, leaving the anterior end of the animal V-shaped with the point anterior. This prevents the anterior surfaces of the two half-tails from fusing and inevitably produces an unusually large amount of anteriorly exposed wound surface. These animals, nevertheless, developed both anterior and posterior heads. These observations do not support the idea of an inhibitory effect of a large anterior surface on the formation of a posterior head.

3. According to Child's theory as applied to planaria, a head always forms at the highest point of a *gradient system*. The relative position of indifferent regenerative material in such a system (and not special head-forming cells or head-inducing or head-localizing agents at the cut surface) determines the formation of a head from the regeneration bud. "As regards relations to the rest of the piece, the reconstitution of a head appears to be primarily a self-differentiation" (Beyer and Child, 1930, p. 358).

The head, which is the first structure to regenerate, soon assumes "physiological dominance" or inductive properties over the structures which regenerate later, and in some instances also over regulative processes in the old tissue.

In planaria an anteroposterior and a mediolateral gradient have been established by Child and his co-workers. In our experiment the crotch head is the anteriormost point on the longitudinal cut surface and at the same time is in the median plane; it would therefore be a point of very high activity both in the anteroposterior and in the mediolateral gradient systems, and thus a preferential locus for head formation. It remains to be explained why a crotch head develops so close to the anterior, which might be expected to exert "physiological dominance" over this region on account of its more anterior position. One would have to assume that the crotch is so highly stimulated that it acquires a physiological level equal to that of the anterior cut surface (or of the intact head in the original experiment of van Duyne). In this respect the explanation is similar to that given by Child for polar heteromorphoses in short transverse pieces (Child, 1915, pp. 98 f.).

On the basis of gradient systems it is difficult to account for the single and multiple lateral heads found in association with crotch heads in Experiment 1 (see also van Duyne, Pl. 10, Fig. 4, and Beissenhirtz, Fig. 18) and, usually, in the absence of crotch heads in Experiments 2 and 3. If the crotch head is the highest point on the longitudinal cut surface, why should a head appear at any other point on this surface? It is true that many of these lateral heads develop near the crotch, but in several cases the heads were formed at a much more posterior level (see Pl. I, Figs. 10 and 11; also Beissenhirtz, Fig. 18). In the 2 cases obtained at Cold Spring Harbor, head formation was activated along most of the longitudinal cut surface (Fig. 4).

An explanation on the basis of gradients would make it necessary to assume alterations or (in the Cold Spring Harbor cases) practically complete obliteration of the anteroposterior gradient along the longitudinal cut surface. It is unlikely that such a change should occur as a result of one median cut. At any rate, it would be an exceptional disturbance of the gradient system, for lateral heads are infrequent in our experiments of Type 1 and have never been reported in experiments in which planarians were split into two separate longitudinal halves.

The experiments of Morgan (1898) and of Beyer and Child (1930) in which lateral heads did regenerate from median surfaces perpendicularly to the main axis cannot be compared with our cases, because these investigators studied head formation in very short lateral pieces. In these short pieces with three cut surfaces lying close together, ". . . . conditions at the anterior and the median cut surfaces are apparently so nearly balanced that in some cases the one, in some cases the other, becomes the determining factor" (Beyer and Child, 1930, p. 360). A corresponding explanation cannot be applied to our experiment because the cutting in the median plane exposes two long surfaces which are in continuity at the crotch and at no point disturbed by a transverse incision.

Therefore, we are led to believe that factors other than gradients come into play in determining the formation of these aberrant lateral heads. Such factors must be sought in the structures of the cut surface.

4. Of the structures exposed at the cut surfaces, the *nervous system* has frequently been regarded as a factor in head determination. In *E. tigrina* (*P. maculata*), which we have used in our experiments, this question has been investigated by Child and coworkers (notably Beyer and Child, 1930; Watanabe, 1935). These investigators came to

the conclusion that the nervous system plays a double role in head localization. When the main cords are cut twice, the posterior section of the cords releases an inhibiting agent which travels anteriorly, reduces activities at the anterior cut surface, and thus lowers the head frequency.

On the other hand, it has been suggested that the central ends of main nerve cords or of lateral branches exert a stimulating effect on head formation. This action would be nonspecific in the sense that the exposed nerve cord helps to maintain or accentuates the high activity of the cells of the cut surface and thus assists in creating a "preferential locus for head formation" (Beyer and Child, 1930).

In our experiments the inhibitory effect cannot come into play at the anterior cut surface because the main nerve cords are cut at the anterior surface only. No main nerve cords are exposed at the longitudinal surface.

A stimulating effect by the nervous system may be involved, however. The anterior heads orginate in the presence of cut ends of the main nerve cords, which are otherwise undisturbed. The posterior (crotch) heads develop at a surface where strong lateral branches undoubtedly are exposed. The localization of lateral heads might be correlated with the exposure of other lateral branches or with concentration of nervous tissue due to muscular contraction. A careful study of sections at the time of origin of crotch heads and lateral heads would give information on this point.

5. The role of the nervous system in the *localization of lateral heads* is hypothetical in the absence of crucial experimental evidence, and at present we can offer no other explanation for their appearance. However, Experiment 2 is of particular interest in this respect, in that it throws light on certain special conditions which favor lateral head formation.

It was found that lateral heads appeared much more frequently, and usually in the absence of crotch heads, when a longitudinal cut was made through tails which were in the process of regeneration. When ordinary tails were split (Expt. 1), 10 out of 100 cases developed lateral heads in addition to crotch heads; but when these half-tails were allowed to regenerate for 8 days and were then split again longitudinally (Expt. 2), 24 out of 28 formed lateral heads. It will be remembered that the second cut was made in the old, pigmented tissue but very near the site of the original cut. The slight deviation of the plane of cutting between the first and second cuts probably is not responsible for the difference in the results. When the tail was allowed to differentiate further (16 days, Expt. 3) and then cut in the same plane as in Experiment 2, the original ratio of lateral to crotch heads was approximated, 5 out of 20 cases forming lateral heads.

Strangely enough, in Experiment 2, no such lateral heads appeared on tails 2 and 3, which consisted almost entirely of newly regenerated tissue (Fig. 4). It seems as though the presence of the regenerating tissue "conditions" the old tissue for lateral head formation, whereas neither old tissue alone nor regenerating tissue alone shows this property to any extent.

It would be premature to attempt an explanation of this situation. However, it should be pointed out that the plane of the second cut in Experiment 2 coincides with the border between old and new tissue. This level is probably more complex in its structural configuration than the adjacent parts of the body, for it is the gateway for regenerative material migrating in a transverse direction—it is the region where the old and the regenerating organ systems (nervous system, intestine, etc.) make connections, etc. It is conceivable that at certain points of this level there exist local conditions which are

particularly favorable for head formation and that these latent potencies may be activated by the second cut.

A further analysis will have to show to what extent the gradient systems, the nervous system, and other local structural or nonstructural conditions are concerned in the formation of lateral heads.[1]

SUMMARY

1. One hundred specimens of *E. tigrina* were split in the median plane to a point between the auricles and the anterior end of the pharynx, and the head was removed by a transverse cut in front of the crotch. In 79 cases regeneration resulted in the formation of two heads, one at the anterior surface and one in the crotch, in addition to the lateral regeneration of the two half-tails. Arguments are presented for the interpretation of this duplication as the Schultze type of duplicitas cruciata.

2. Occasional absence or subnormal development of heads was correlated with contraction or fusion of the cut surface, which resulted in a lack of exposed tissue.

3. In 10 cases, one or two lateral heads formed in addition to the crotch head; in 7 cases, oversized heads with three or four eyes formed in the crotch.

4. When the trunks and tails of the duplicitas cruciata were split again, 8 days after the first operation, the percentage of lateral heads was greatly increased and that of crotch heads was decreased.

5. The lateral heads developed only on the half-tails which consisted of old tissue and never on the half-tails which consisted mainly of regenerated tissue.

6. When the trunks and tails of the duplicitas cruciata were split 16 days after the first operation, crotch-head formation was again predominant, as in the first experiment.

7. The role of gradient systems, of the nervous system, and of other possible factors involved in the localization of heads are discussed.

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PLATE I

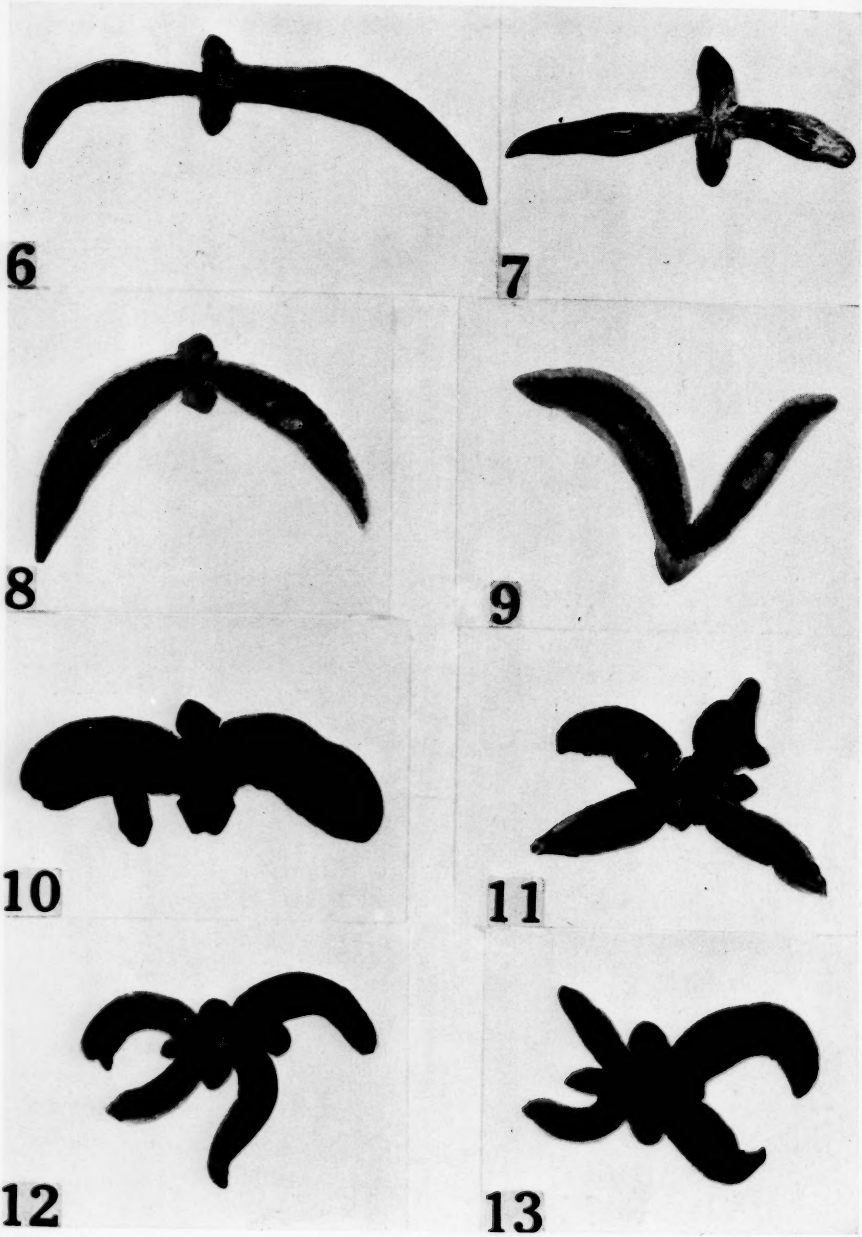

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PLATE I

FIGS. 6–7 and 10–13.—Photographs of animals fixed in Zenker's solution and stained with Ehrlich's hematoxylin.

FIG. 6.—Experiment 1: duplicitas cruciata several weeks after the operation of Experiment 1. 5×.

FIG. 7.—Same. Double pharynx on right side. 5×.

FIG. 8.—Experiment 1: duplicitas cruciata, 16 days after operation. Animal fed a mixture of trypan blue and egg yolk. Note lateral position of pharynges as a landmark for the regenerating side of the tails. 8×.

FIG. 9.—Experiment 1: 16 days after operation, anterior head absent. 8×.

FIG. 10.—Experiment 1: duplicitas cruciata, 8 days after operation. Duplication of crotch head, formation of a lateral head, and duplication of both pharynges. 8×.

FIG. 11.—Experiment 2: 10 days after operation as shown in Figure 1, *c*. Formation of two lateral heads on tail 1. 8×.

FIG. 12.—Experiment 2: 6 days after operation; one anterior head, two crotch heads, and one head slightly lateral (its lateral position was unmistakable on the living animal). 8×.

FIG. 13.—Experiment 3: 8 days after operation; one anterior head, two normal crotch heads, and one subnormal crotch head. 8×.

RESPIRATORY METABOLISM DURING LARVAL AND PUPAL DEVELOPMENT OF THE FEMALE HONEYBEE (APIS MELLIFICA L.)

(Five figures)

R. M. MELAMPY AND E. R. WILLIS[1]
U.S. Department of Agriculture, Bureau of Entomology and Plant Quarantine, and Department of Zoölogy, Louisiana State University

THE present study is an investigation of the respiratory exchange accompanying the differentiation of female larvae of the honeybee (*Apis mellifica* L.) into queens and workers. The honeybee affords an unusual opportunity for measuring the respiratory metabolism of a larval insect, as honeybee larvae are relatively inactive. Insects, although able to live anaerobically for a short time, derive their energy from oxidation. Therefore, the respiratory exchange of insects is of significance since it provides an index to the rate of metabolism. The ratio between the carbon dioxide production and oxygen consumption is known as the "respiratory quotient" (R.Q.), and, as Richardson (1929) has stated, it "is the most direct measure, so far devised, of the relative quantity of foodstuffs entering into the metabolism of the human or animal organism."

The results obtained by Melampy, Willis, and McGregor (unpublished) from a biochemical study of the growth of the reproductive and worker castes of the female honeybee indicate that differences in metabolic activity are associated with caste differentiation. The queen, or reproductive caste, is characterized in the larval stage by a more rapid growth as well as by a higher nitrogen, lipid, reducing substance, and energy content, as compared with the worker caste. Since the beginning of the nineteenth century, biologists have suggested that the difference in nutrition of the castes determines whether a female larva of the honeybee will develop into a queen or a worker. During the first three days of larval life the female larvae receive royal jelly. After this period the reproductive caste continues to receive this diet, whereas that of the worker caste is altered.

The writers are indebted to Dr. T. C. Evans, of the State University of Iowa, and Dr. E. J. Boell, of Yale University, for their helpful suggestions.

REARING OF INSECTS

From eight to ten colonies of Italian bees were used to produce the workers for this study. In order to have available larvae and pupae of a known age, a queen was caged on a worker brood comb. After 24 hours the queen was removed, and the comb containing the eggs was dated and placed in the brood nest. Further oviposition on numbered combs was prevented by excluding the queens.

Since the queen was caged for 24 hours on the brood frame, it is apparent that there would be a 24-hour age range for the larvae. In Tables 1, 2, and 3 the limits of age range are presented; for example, the 0–1 entry under "Age (Days)" in Table 1 means that the oldest larvae would be 1 day old and the youngest would have just hatched.

[1] Graduate Fellow, Louisiana State University.

The queens were produced by transferring newly hatched female larvae to queen cells and allowing the larvae to be reared in queenless colonies.

The average live weight per individual insect was obtained by weighing representative groups of insects at 24-hour intervals during larval and pupal development. The insects were weighed in groups of 25 to 150 individuals, depending upon the stage of development. This was necessary since it was observed that considerable variation existed between the chronological age and physiological and morphological development. Each value in Table 1 represents the average weight of at least 450 individuals and not more than 900.

The results presented in Table 1 for the growth of the larval worker honeybee are in agreement with the data for the same caste published by Nelson, Sturtevant, and Lineburg (1924), except that the average weight at the 4–5-day stage is lower.

TABLE 1

AVERAGE WEIGHTS OF QUEEN AND WORKER HONEYBEES AT 24-HOUR INTERVALS DURING THE LARVAL AND PUPAL STAGES

Age (Days)	Live Weight of Queens (Milligrams)	Live Weight of Workers (Milligrams)	Age (Days)	Live Weight of Queens (Milligrams)	Live Weight of Workers (Milligrams)
0–1	.....	0.20	10–11	243.47	135.63
1–2	0.90	1.12	11–12	215.38	134.53
2–3	10.55	6.08	12–13	195.54	131.31
3–4	44.93	25.38	13–14	.....	124.42
4–5	133.24	96.64	14–15	.....	126.67
5–6	256.56	143.61	15–16	.....	126.12
6–7	262.34	136.89	16–17	.....	124.16
7–8	249.70	135.56	17–18	.....	122.71
8–9	249.60	134.21	18–19	.....	113.36
9–10	242.05	130.65			

RESPIRATORY MEASUREMENTS

The queen emerges as an adult in approximately 13 days after the hatching of the larva, and the worker in 18 days. For respiratory measurements the queens were selected up to 11–12 days of development, and the workers up to 16–17 days.

The Barcroft-Warburg technique was used to obtain the data on oxygen consumption and carbon dioxide production. All measurements were made on individual insects at $35^\circ \pm 0^\circ.02$ C. A larva or pupa was placed in the well of the Warburg flask, and 10 per cent potassium hydroxide or water was placed in the main chamber, according to whether oxygen consumption or carbon dioxide production was being measured. The oxygen consumption and the carbon dioxide production were measured on the same insect with the same manometer by first determining the manometer change resulting from the simultaneous oxygen uptake and carbon dioxide production. From this combined manometer change the carbon dioxide production rate was determined algebraically by adding the oxygen consumption rate determined in a succeeding equivalent period of time. Through a series of preliminary determinations it was found that the respiratory exchange was the same in both periods; thus, the values obtained could be used in calculating the respiratory quotient.

The average oxygen consumption and carbon dioxide production per gram of insect tissue were determined by the formulas

$$\text{Oxygen consumed} = \frac{h \cdot k\text{O}_2}{wt} \cdot 1{,}000\,,$$

$$\text{Carbon dioxide produced} = \frac{h \cdot k\text{CO}_2}{wt} \cdot 1{,}000\,,$$

where the oxygen or carbon dioxide is expressed as cubic millimeters per gram; h is the manometer reading for 1 hour corrected for thermobarometric changes, and in the case of the carbon dioxide representing the difference between the oxygen consumption and the carbon dioxide production; $k\text{O}_2$ and $k\text{CO}_2$ are the manometer constants for the respective gases; wt is the live weight of the insect in milligrams; and 1,000 is the number of milligrams per gram. The reader is referred to Dixon (1934) for further details as to procedure.

The values obtained for normal carbon dioxide production should be correct as determined by the Barcroft-Warburg procedure; it was assumed that the body fluids of the larvae and pupae of the honeybee are saturated with respect to carbon dioxide for the existing physiological conditions. Needham (1932, 1933) has criticized the use of the direct method for the determination of respiratory quotients, because of the possibility that some of the carbon dioxide produced during metabolism may be retained by tissues. Boell (1935), working with eggs of *Melanoplus differentialis* (Thos.), found a small amount of bound carbon dioxide during embryonic development. Attempts were made to determine the bound carbon dioxide in both larval and pupal honeybees by adding 3N hydrochloric or sulphuric acid to the intact organism in the respiration flask, but the integument of the insects was apparently impermeable to the acids. No measurable quantity of bound carbon dioxide was obtained from tissue brei of larval or pupal honeybees. The manometers were shaken at the rate of approximately one hundred complete oscillations per minute during these determinations. Bishop (1923) reported a pH value of 6.8 for honeybee larva blood and suggested that the increased hydrogen-ion concentration during metamorphosis favors histolysis. Taylor, Birnie, Mitchell, and Solinger (1934), as well as other investigators, have found the body fluids of insects to be acid. If any bound carbon dioxide existed, it would probably be in the form of carbonic acid. The results of these determinations are presented in Table 2.

The maximum oxygen consumption and carbon dioxide production for both castes occur during early larval life at the time of rapid growth. This period is characterized by the rapid synthesis of the structural proteins, lipogenesis, and a storage of carbohydrate reserves. With the onset of pupation the rate of metabolism is reduced to a minimum, to be followed by a rise prior to emergence which nearly equals that in the early stage. To facilitate a comparison between the respiratory metabolism of the two castes, the data in Table 2 are presented graphically in Figures 1 and 2.

The reader's attention is called to the fact that at a given time the queens are more advanced with respect to morphological differentiation than the workers; for example, the queens pupate between the fifth and sixth days, whereas the workers do not transform until approximately the eighth day. There is a similarity in the curves, although the rate of metabolism is higher for the queens throughout the larval and pupal stages. The

marked reduction in respiratory rate per gram of tissue occurs during the period of the most rapid increase in body weight. Observations made on various vertebrates and invertebrates indicate that the growth-rate decreases from birth to maturity and that the greatest decrease is found early in life. In the early stages of animal development the

TABLE 2

AVERAGE OXYGEN CONSUMPTION AND CARBON DIOXIDE PRODUCTION OF QUEEN AND WORKER HONEYBEES PER UNIT WEIGHT OF TISSUE PER HOUR

Age (Days)	Number of Insects	Oxygen Consumed		Carbon Dioxide Produced	
		Mean Volume (Cubic Millimeters per Gram per Hour)	Coefficient of Variation (Per Cent)	Mean Volume (Cubic Millimeters per Gram per Hour)	Coefficient of Variation (Per Cent)
	Queens				
2–3	21	4,554 ± 166	16	5,208 ± 244	21
3–4	20	3,423 ± 83	10	4,130 ± 138	14
4–5	20	2,446 ± 68	12	3,150 ± 104	14
5–6	19	1,391 ± 83	26	1,336 ± 106	34
6–7	21	760 ± 13	8	671 ± 11	7
7–8	27	721 ± 7	5	634 ± 9	7
8–9	22	718 ± 11	7	617 ± 11	8
9–10	23	873 ± 28	15	728 ± 28	18
10–11	23	1,162 ± 16	6	965 ± 12	6
11–12	23	1,464 ± 34	11	1,214 ± 31	12
	Workers				
2–3	58	2,901 ± 74	19	4,121 ± 94	17
3–4	55	2,238 ± 43	14	2,746 ± 58	15
4–5	59	1,063 ± 22	15	1,308 ± 36	21
5–6	57	627 ± 20	24	710 ± 22	24
6–7	56	409 ± 6	12	425 ± 6	11
7–8	57	374 ± 4	9	384 ± 5	11
8–9	56	396 ± 11	21	417 ± 10	19
9–10	64	414 ± 3	5	401 ± 4	8
10–11	57	415 ± 3	6	399 ± 4	9
11–12	58	466 ± 5	8	445 ± 5	9
12–13	57	509 ± 4	7	482 ± 5	8
13–14	59	629 ± 6	8	593 ± 8	10
14–15	58	754 ± 6	6	692 ± 7	8
15–16	56	818 ± 12	11	759 ± 13	13
16–17	65	1,025 ± 16	13	963 ± 18	15

chemical reactions are such as to bring about the synthesis of compounds which are to serve for structural and reserve purposes, such as proteins, fats, and carbohydrates.

The rates of oxygen consumption and carbon dioxide production per individual insect were obtained by multiplying the average weight, as given in Table 1, by the average rates of oxygen consumption and carbon dioxide production for the same age, as given

in Table 2. These figures have been plotted in Figures 3 and 4. The similarity in the development of the queens and workers and the more rapid rate of respiratory exchange of the reproductive caste shown in Figures 1 and 2 are again evident. However, when

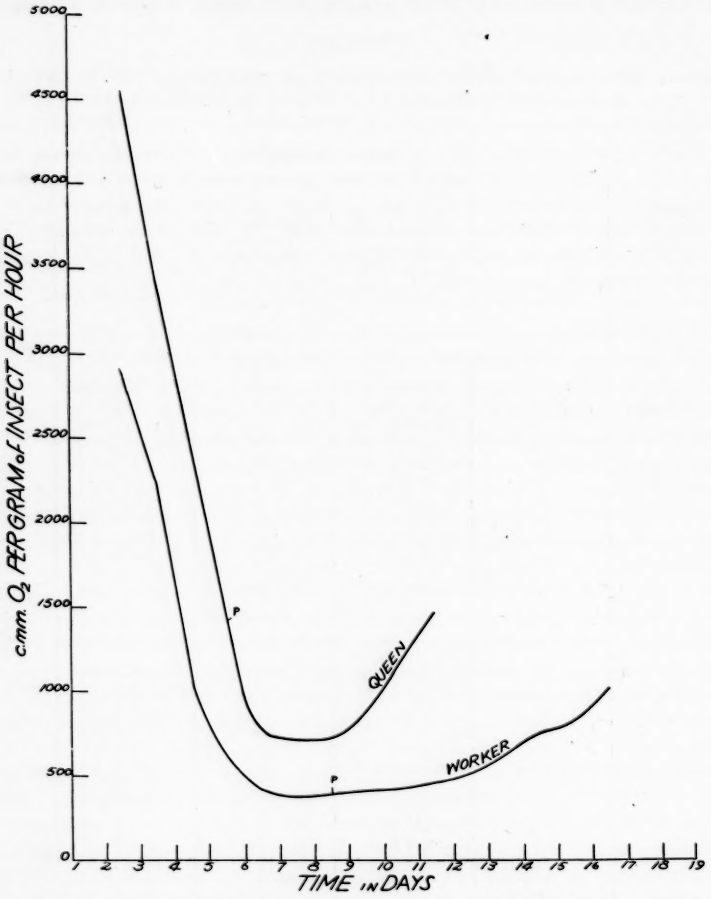

FIG. 1.—Oxygen consumption per gram of insect tissue per hour during larval and pupal development of the queen and worker honeybees. *P*, time of pupation.

calculated on an equivalent basis (per gram of tissue per hour), the rate of respiratory metabolism decreased during the larval period, whereas it showed an increase when calculated on the basis of an individual insect because of the increase in mass. The increase in respiratory rate prior to emergence is related to the development of the imaginal disks which are formed early in larval life and wait until the pupal stage before they start growing.

The pronounced physiological effect of the change in the diet on the worker is clearly demonstrated. It is of considerable interest that the queen pupated at an oxygen consumption of 355 cu. mm. per insect per hour, whereas the worker transformed at an oxy-

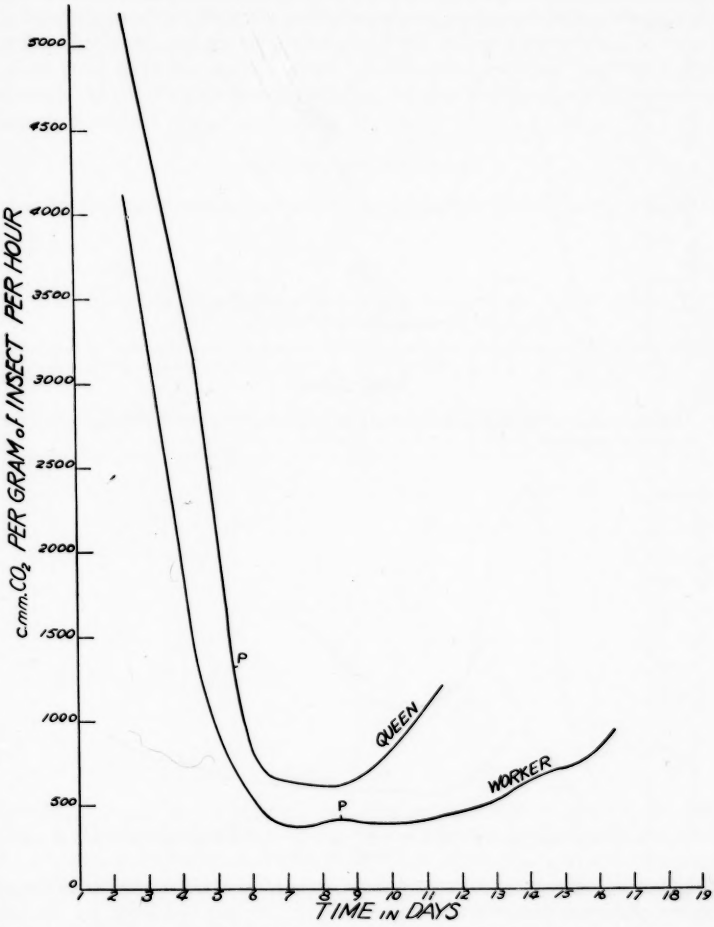

FIG. 2.—Carbon dioxide production per gram of insect tissue per hour during larval and pupal development of the queen and worker honeybees. *P*, time of pupation.

gen consumption of 50 cu. mm. per insect per hour. The queen weighed, on an average, 256 mg. at the time of pupation; and the worker, 134 mg.

It is evident that differentiation between the two castes is well under way by the second day of larval life. In the experimental procedure used, the newly hatched larvae

(less than 12 hours old) were placed in queen cells in queenless colonies, and the larvae were supplied with more food than larvae of the corresponding age in worker cells; hence the accelerated development. Figures 3 and 4 demonstrate that physiologically the members of the worker caste begin postembryonic development with the capacity to grow

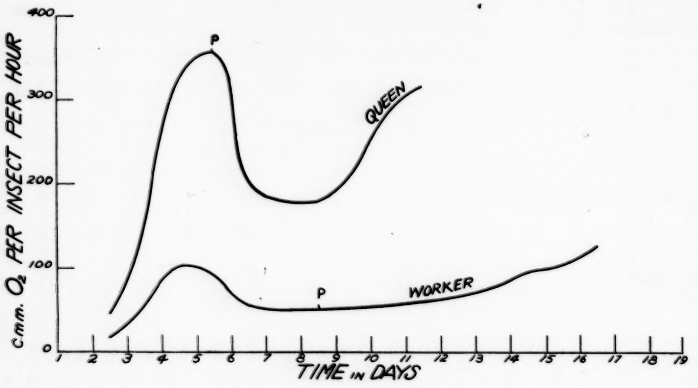

FIG. 3.—Oxygen consumption during larval and pupal development per individual queen and worker honeybee. *P*, time of pupation.

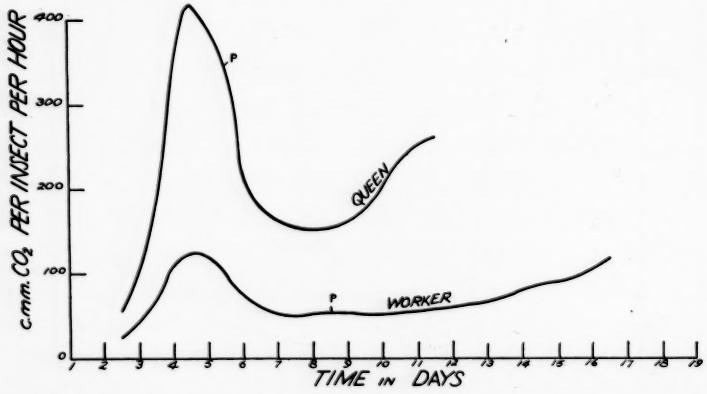

FIG. 4.—Carbon dioxide production during larval and pupal development per individual queen and worker honeybee. *P*, time of pupation.

similar to that of larvae which develop into the queen caste. Figures 3 and 4 indicate further that the growth per individual is accompanied by an increased respiratory exchange during larval and late pupal development. Zander and Becker (1925) found, in their work on the influence of larval age upon the development of the organs of the queen, that the critical age was between 78 and 90 hours. Queens developing from these individuals were of a transition type. Larvae over 90 hours old produced only workers.

Several workers have investigated the respiratory metabolism of other species of insects during the pupal stage and have found a typical U-shaped curve, indicating that oxygen consumption and carbon dioxide production are high at the beginning, decrease, and then rise to a maximum. This general finding is in agreement with the results presented in Figures 3 and 4. The curves for the worker caste do show a decline in the rate of metabolism following the larval period and a rise before emergence, but the change in rate is not as great as in the queen curves. The reader is referred to Fink (1925), Taylor and Steinbach (1931), Bodine and Evans (1932), and Poulson (1935) for summaries of the earlier work in this field.

RESPIRATORY QUOTIENTS

The respiratory quotients calculated from the data in Table 2 are presented in Table 3 and Figure 5.

TABLE 3

RESPIRATORY QUOTIENTS DURING LARVAL AND PUPAL DEVELOPMENT OF THE QUEEN AND WORKER HONEYBEES

Age (Days)	Queens		Workers	
	No. of Insects	R.Q.	No. of Insects	R.Q.
2–3	21	1.14	58	1.42
3–4	20	1.21	55	1.23
4–5	20	1.29	59	1.23
5–6	19	0.96	57	1.13
6–7	21	0.88	56	1.04
7–8	27	0.88	57	1.03
8–9	22	0.86	56	1.05
9–10	23	0.83	64	0.97
10–11	23	0.83	57	0.96
11–12	23	0.83	58	0.95
12–13	.....	.....	57	0.95
13–14	.....	.....	59	0.94
14–15	.....	.....	58	0.92
15–16	.....	.....	56	0.93
16–17	.....	.....	65	0.94

The respiratory quotient is of physiological significance as an index to the quality and quantity of the substances oxidized during metabolism. For example, a respiratory quotient of 1.0 indicates the oxidation of carbohydrate, since one molecule of carbon dioxide is produced for each molecule of oxygen consumed. The respiratory quotient for fat is 0.70, as lipids are relatively poor in oxygen, and in the catabolism of these substances by the animal organism some of the oxygen consumed appears as water. The volume of carbon dioxide produced by the oxidation of fat is smaller than the volume of oxygen consumed; thus the ratio is less than 1. The average respiratory quotient for protein is 0.80. Low quotients may be produced by the conversion of protein and fat into carbohydrate or by the incomplete oxidation of metabolites, whereas high respiratory quotients (greater than unity) indicate the formation of fat from carbohydrate. According to Krogh (1916), the usual respiratory quotients for animals lie between 0.72 and 0.97.

However, chemical reactions yielding high or low quotients may proceed to a limited degree, and the respiratory quotient remain within its usual range.

The respiratory quotient is greater than 1 during the larval stages for both castes of the honeybee, indicating a synthesis of lipid from carbohydrates which had been previously demonstrated by chemical analysis (unpublished). The increased production of carbon dioxide during this period is due to the synthesis of oxygen-poor fats from carbohydrates which contain relatively more oxygen. The oxygen liberated during the fat synthesis is available for oxidation and will replace the same amount of absorbed oxygen in catabolic processes, and consequently the respiratory quotient will rise.

Wierzuchowski and Ling (1925) reported respiratory quotients of 1.4 and 1.58 for fattening pigs that had been fed starch. Bleibtreu (1901) observed quotients up to 1.38

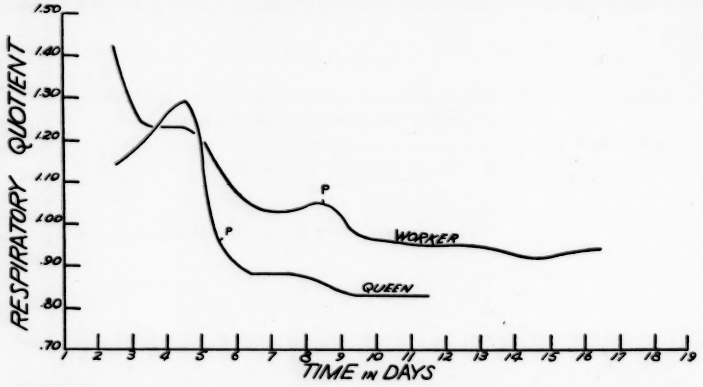

FIG. 5.—Respiratory quotients of queen and worker honeybees during larval and pupal development. *P*, time of pupation.

for fattening geese. Krogh (1916) has suggested that a considerable amount of carbon dioxide is produced by fermentation in the gut of certain species of animals, thus producing an increase in the respiratory quotient that is not an index of the metabolic processes. McCleskey and Melampy (1938) found that royal jelly of the honeybee has bactericidal properties. This may or may not affect bacterial flora in the gut of this insect, which in turn might influence the production of carbon dioxide. Cook (1932) reported the respiratory quotient of both normal and defaunated termites (*Termopsis nevadensis*) as approximately unity, but in starved individuals the respiratory quotient was low.

SUMMARY

Measurements of oxygen consumption and carbon dioxide production have been made on individual members of the queen and worker castes of the honeybee (*A. mellifica* L.) throughout larval and pupal development. The respiratory rates rise per individual insect during the larval period, fall to a minimum during pupation, and finally rise before emergence.

The queen or reproductive caste is characterized by a higher metabolic rate, which is associated with the more rapid growth-rate of this caste.

Respiratory quotients greater than unity indicate a synthesis of lipid from carbohydrate for both castes during the larval stages.

The respiratory quotient for the queen ranges between 0.96 and 0.83 during the pupal stage, whereas that for the worker ranges between 1.05 and 0.94. The average respiratory quotient for the queen during the pupal stage is 0.87, whereas that for the worker is 0.96.

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A DETERMINATION OF THE TEMPERATURE COEFFICIENTS FOR OXYGEN UPTAKE OF NORMAL AND BLOCKED EMBRYOS AND EGGS (ORTHOPTERA)[1]

JAMES P. GOODRICH AND JOSEPH HALL BODINE
Zoölogical Laboratory, State University of Iowa

WHILE the effects of temperature on biological systems have been investigated by many workers (see monograph by Belehradek, 1935), our knowledge of the effects of temperature on respiratory mechanisms is very vague. Since a great deal of physiological data is available concerning diapause and developing grasshopper eggs and embryos (Bodine, 1925, 1929, 1932, 1934, and Bodine *et al.*, 1934–38), it was thought desirable to investigate the temperature relations involved in the rate of oxygen consumption and to compare the responses of eggs with those of their contained embryos.

Rubenstein and Gerard (1934) have found that the Q_{10} for oxygen consumption of the unfertilized *Arbacia* egg is about twice that of the fertilized egg, and Korr (1937) has experimentally altered the respiratory rates and mechanisms of fertilized and unfertilized eggs of the same species and measured the rates at different temperatures. In view of certain similarities in the respiratory mechanisms of *Arbacia* and grasshopper eggs, a comparison of their temperature relations has been undertaken.

MATERIALS AND METHODS

Eggs of the grasshopper, *Melanoplus differentialis*, having a known temperature history and of known chronological and morphological ages, were used throughout the experiments. For the study of prediapause and diapause, eggs were placed on moist sand in a constant-temperature chamber at 25° C. (in the pods in which they were laid and not more than 24 hours after laying) until the proper age had been attained. In this paper, age of eggs or embryos is expressed as days at 25°, since Slifer (1932) has described the embryos of this species in those terms. Two thousand to eight thousand eggs or embryos constituted a series of experiments, and about twelve thousand eggs or embryos were used in all.

Diapause, a period of quiescence which eggs of this species enter at about the end of the third week of development at 25°, can be broken by subjecting them to constant low temperature for 2 months or more (Bodine, 1925, 1929). Consequently, eggs for use in the study of postdiapause were allowed to develop at 25° until well into diapause and then removed to a temperature of 5° for a period of time sufficient to break diapause. When again placed at 25°, they were allowed to develop to the 5-day postdiapause stage, by which time blastokinesis had been completed and yolk engulfment begun (Slifer, 1932). Eggs for the study of diapause were kept at 25° until they were at least 50 days in diapause.

When the eggs had reached the desired chronological age, they were removed from the pods, washed free of debris in distilled water, sterilized in 70 per cent alcohol for 10 minutes, and washed again several times in sterile distilled water. Embryos were dis-

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sected out in sterile Belar (modified Ringer) solution buffered to pH 6.3 with M/15 phosphate, and washed free of adhering yolk cells. The embryos were examined carefully, and only those of the correct morphological age were used.

Oxygen consumption was measured in microdifferential manometers, and different numbers of eggs or embryos were run in each manometer, depending on the age: 25–35 prediapause and diapause eggs or embryos were run in each manometer, and 8–10 postdiapause embryos were used. One cubic centimeter of sterile buffered Belar solution, including embryos, was used in each manometer; and the carbon dioxide produced in their respiration was removed by a small roll of filter paper soaked with 0.1 cc. of 10 per cent KOH placed in a small glass cup suspended in the reaction chamber of the manometer. When eggs were run, they were added to 1.0 cc. of sterile Belar solution.

The manometers were shaken at a rate of 75–80 oscillations per minute, which was found to be fast enough to permit adequate gas exchange without injuring the embryos. Three water baths were used, which were held constant to within $\pm 0.2°$ at 15°, 25°, and 35°, respectively. Readings were taken at 15- or 20-minute intervals over a period of 1–2 hours at each temperature, and 15 or 20 minutes were allowed in each case for temperature equilibration with the manometer taps open. Except for those in the first series of experiments, eggs or embryos were run consecutively at 15°, 25°, and 35°. At the end of each experiment the embryos were again examined, and the pH of the medium was checked by colorimetric estimation with bromothymol blue.

Three temperature coefficients were calculated: the thermal increment, or temperature characteristic, μ; Belehradek's temperature coefficient, b; and the Q_{10}. The Q_{10} in each case was calculated from the values for oxygen consumption found in each experiment, and each value listed for Q_{10} is the mean for several experiments. The thermal increment and b were calculated from the mean values of the oxygen consumption (as mm.^{3} O_2/egg or embryo/hour) at 15°, 25°, and 35°. The values of μ were calculated from the familiar van't Hoff–Arrhenius equation

$$\mu = \frac{4.6\,(\log K_2 - \log K_1)}{\frac{1}{T_1} - \frac{1}{T_2}}, \tag{1}$$

where K_1 and K_2 are the velocities at temperatures T_1 and T_2, respectively, the temperature being taken in degrees of the absolute scale. The Q_{10} rule, though originally derived from a formula of Berthelot (1862), may be stated as a simplification, for small temperature intervals, of the van't Hoff–Arrhenius law. Values for this coefficient were calculated from the equation

$$Q_{10} = \left(\frac{K_2}{K_1}\right)^{10/(t_2 - t_1)}. \tag{2}$$

Belehradek (1931, 1935) has proposed an empirical temperature equation where the temperature coefficient b is calculated from the equation

$$b = \frac{\log v_2 - \log v_1}{\log t_2 - \log t_1}, \tag{3}$$

v_1 and v_2 being velocities at temperatures t_1 and t_2, respectively.

RESULTS

Three series of experiments were carried out, the results of which are summarized in Tables 1 and 2. Table 1 lists the values found in the three series for all coefficients. In Series I the oxygen consumption of diapause and postdiapause embryos was measured, using the same temperature interval but a different sequence of temperatures (i.e., from 25° to 35° or from 35° to 25°). Since the values of all coefficients, except b for diapause embryos, vary when a different temperature sequence was used, in all the following experiments runs were started at the lower temperature in order that coefficient values might be comparable.

In the second series, diapause, postdiapause, and 15- and 18-day prediapause embryos were used. As is found with most processes, the temperature coefficients decrease

TABLE 1

TEMPERATURE COEFFICIENTS OF EGGS AND EMBRYOS

	15°–25° C.				25°–35° C.				35°–25° C.			
	No. of Expts.	Q_{10}	μ	b	No. of Expts.	Q_{10}	μ	b	No. of Expts.	Q_{10}	μ	b
Series I:												
Diapause	.....	.....	.....	.....	15	1.71	6,954	1.21	9	1.98	11,107	1.19
Postdiapause	.....	.....	.....	.....	18	1.09	1,570	0.26	4	1.28	4,314	0.70
Series II:												
Diapause	17	1.87	9,221	1.06	17	1.79	9,606	1.56	.....	.....	.....	.....
Postdiapause	15	2.33	14,178	1.63	15	1.08	908	1.47	.....	.....	.....	.....
Prediapause:												
15 days	10	2.52	15,663	1.78	10	1.65	10,207	1.66	.....	.....	.....	.....
18 days	9	2.93	18,349	2.10	6	1.67	6,956	1.13	.....	.....	.....	.....
Series III:												
Prediapause												
19-day egg	4	1.82	9,598	1.10	4	1.30	4,838	0.79	.....	.....	.....	.....
19-day embryo	4	2.50	14,705	1.69	4	1.69	9,513	1.54	.....	.....	.....	.....
Diapause:												
Eggs	22	1.82	9,364	1.07	23	1.47	5,034	0.82	.....	.....	.....	.....
Embryos	16	2.12	12,768	1.46	18	1.94	12,168	1.30	.....	.....	.....	.....

with increasing temperature, although Korr (1937) found that with *Arbacia* eggs the Q_{10} increases with increasing temperature. In diapause embryos the values of μ and Q_{10} remain at about the same level, while b increases at the higher temperature range. Since the diapause embryo shows lower temperature coefficients than the developing one, it is apparently less sensitive to these temperature changes, and it might be expected that its temperature coefficients would be more constant at different temperatures. It is interesting that at 15°–25° the highest values for temperature effects on prediapause embryos occur at 18 days, when the oxygen consumption at 25° is greatest (Bodine and Boell, 1936*c*); whereas at 19 days, when oxygen consumption begins to decline, just before the beginning of diapause, the temperature coefficients also are lower. Explanation cannot readily

be given for the very low temperature coefficients of postdiapause embryos at the higher temperature range, both in those which were run only at 25° and 35° and those run at 15°, 25°, and 35°. Temperatures as high as 35° certainly do not have injurious effects, since Bodine (1925) has found that hatching-time is decreased when eggs are kept at this temperature.

To compare the temperature coefficients of eggs and embryos, a third series of experiments was performed, using prediapause and diapause eggs and embryos. Eggs and embryos were run on consecutive days, and embryos were always used in the same manometers which were employed for the eggs from which they were dissected. In order that

TABLE 2

MEAN VALUES AND PROBABLE ERROR OF MEAN FOR Q_{10} OF EGGS AND EMBRYOS

	15°–25° C.	25°–35° C.	35°–25° C.
Diapause embryos:			
Series I	.....	1.71±0.16	1.98±0.12
Series II	1.87±0.08	1.79±0.07	.....
Series III	2.12±0.11	1.94±0.08	.....
Mean	1.99±0.07	1.82±0.06	.....
Prediapause embryos:			
Series II:			
15 days	2.52±0.13	1.62±0.07	.....
18 days	2.93±0.17	1.67±0.13	.....
Series III:			
19 days	2.50±0.27	1.69±0.03	.....
Mean	2.68±0.10	1.67±0.05	.....
Postdiapause embryos:			
Series I	.....	1.09±0.05	1.28±0.06
Series II	2.33±0.08	1.08±0.05	.....
Mean	.....	1.08±0.04	.....
Diapause eggs:			
Series III	1.82±0.07	1.47±0.02	.....
Prediapause eggs:			
Series III	1.82±0.24	1.30±0.04	.....

eggs and embryos might be run at as nearly the same stage of development as possible, the eggs, after each run, were completely immersed in Belar solution and kept at 25° until the next day, when the embryos were dissected out, since Boell (1935) and Schipper (1938) have found that the development of eggs immersed in unaerated solutions is retarded. It is seen that for a given lot of diapause or prediapause eggs the temperature coefficients of the embryos are larger than those of the eggs from which they were removed.

The mean values and probable error of the mean for the Q_{10} of eggs and embryos are listed in Table 2.

DISCUSSION

It has been shown (evidence reviewed by Korr, 1937) that the respiration of the fertilized *Arbacia* egg proceeds largely through the cyanide-sensitive cytochrome-indophenol oxidase (Warburg-Keilin) system, while that of unfertilized eggs proceeds mainly through cyanide-stable nonferrous carriers. Korr (1937) has reviewed evidence and presented additional data showing that the maximum rate of substrate activation of which the dehydrogenases are capable is the same in fertilized and unfertilized eggs, and hence that the rate-controlling link in the oxidative chain is the hydrogen transfer mechanism. Rubenstein and Gerard (1934) and Korr (1937) find that the temperature coefficient for oxygen uptake of unfertilized eggs is about twice as large as that of fertilized eggs. Korr concludes, then, that "the less closely the rate of hydrogen transfer approaches the rate of substrate activation the larger the temperature coefficient; the more nearly this value is reached, the smaller the coefficient—down to the value found for the fertilized egg."

Bodine and collaborators (1934–38) have studied the effect of cyanide, methylene blue, and carbon monoxide on developing and diapause grasshopper eggs and find that with respect to respiration the developing egg behaves similarly to the fertilized *Arbacia* egg and that the responses of the diapause egg are very much like those of the unfertilized *Arbacia* egg. Their results would seem to suggest that certain of the respiratory mechanisms involved are very much alike in both cases.

At the higher temperature range (25°–35°) the present data are somewhat variable. As shown by Table 1, diapause embryos that are run from 15° to 25° to 35° show coefficients that are about the same as those of 15-day embryos run in the same way. However, 18-day embryos have coefficients which are somewhat smaller than those of embryos in diapause. Diapause embryos run only at 25°–35° show higher Q_{10} and μ values than any of the foregoing, while diapause embryos of the third series show Q_{10} and μ values which are still higher. Nineteen-day embryos of this series show values for all three coefficients which are practically identical with those of diapause embryos in the second series. In view of the variability of the temperature coefficients at this temperature range, certainly they cannot be used as the basis for any definite statements about the respiratory processes involved.

However, in the lower temperature range (15°–25°) the data are more consistent. As shown by Table 2 the temperature coefficients of diapause embryos are considerably smaller than those of either prediapause or postdiapause developing ones. While the respiratory processes of fertilized and unfertilized *Arbacia* eggs do show certain similarities to those of developing and diapause grasshopper eggs, respectively, the fact that fertilized *Arbacia* eggs have lower temperature coefficients than unfertilized ones makes it evident that some aspects of their respiration cannot be the same. It is impossible to determine from these experiments just what the differences may be. The Q_{10} of diapause eggs is about the same as that found by Bodine and Thompson (1935) on diapause eggs used as controls, making allowance for the somewhat different temperature interval (20°–30°) which they employed. Rogers (1929), using that temperature range, obtained a Q_{10} of 2.40 for oxygen consumption of *M. differentialis* nymphs at the second to fourth instar.

Bodine and Boell (1936*c*) have pointed out the necessity for considering problems of metabolic activity during embryonic development not only in terms of the egg as a whole but also in terms of the contained embryo. It is apparent from the data in Table 1 that

this point of view is justified. They have found that the respiratory rate of embryos is lower than that of whole eggs throughout the entire period of embryonic development. Since the respiration of the yolk cells and embryonic cells may not necessarily be governed by exactly the same processes (and it would seem that they are not), it is not surprising that the temperature responses of embryos and whole eggs are somewhat different. The coefficients obtained using the intact egg must be resultant values of the accelerative effect of increased temperature on the yolk cells and extra-embryonic membranes plus the embryonic cells themselves. Although stimulation cannot definitely be ruled out as a possible factor in the higher-temperature coefficients of the embryo, since the resultant values are less both in diapause and prediapause (Table 1) than those for the embryo, it would appear that the yolk cells are not affected by temperature to as great an extent as is the embryo. It is interesting that in prediapause the yolk cells and serosa cells are very resistant to X-rays while the embryo is quite sensitive to them (unpublished data of Dr. E. E. Carothers; see Ray, 1938).

SUMMARY

1. Temperature coefficients for oxygen consumption of *M. differentialis* eggs and embryos of different ages and in different physiological states have been determined at two temperature intervals (15°–25° and 25°–35°).

2. Different values for the coefficients are found when different sequences of experimental temperatures are employed.

3. In the lower temperature range the coefficients of diapause embryos are lower than those of either prediapause or postdiapause developing ones. In the higher temperature range no significant differences between responses of diapause and prediapause developing embryos can be seen. Postdiapause embryos are practically insensitive to temperature changes from 25° to 35°.

4. Diapause and prediapause embryos have higher coefficients than the eggs from which they were removed. The probable significance of this is pointed out.

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RETARDATION OF EARLY CLEAVAGE OF URECHIS BY ULTRA-VIOLET LIGHT

(One plate and one figure)

ARTHUR C. GIESE
Stanford University

ULTRA-VIOLET light is known to have strong effects upon protoplasm and therefore has served as a useful tool in biological investigations. Yet we know comparatively little as to the mode of action of these radiations and so cannot use them most effectively. Quantitative studies of a comparative nature should throw some light on this complex problem. In a preceding study (Giese, 1938*a* and 1938*b*) effects of known dosages of monochromatic ultra-violet light upon the cleavage of sea-urchin eggs were reported. In the following paper a comparable study on the effects of ultra-violet light on the cleavage of the marine worm *Urechis caupo* Fisher and MacGinitie is presented.

The animals used in this study were collected during the spring and summer of 1938 at Bolinas Bay, California. They were transported to the laboratory in precooled food jugs and kept two or three per gallon jar in 500–1,000 cc. aerated sea water (obtained at Bolinas Bay) in a constant-temperature room (15 ± 0°.5 C.). The sea water was changed whenever necessary. Under such conditions the worms remained healthy in many cases for as long as a month.

Eggs and sperm were drawn from the segmental organs with fine pipettes, one organ being entered at a time and each organ only once, a chart being kept for the removal of materials from each animal. In experiments with monochromatic light the eggs and sperm were irradiated at room temperature (20°–23° C.) but were quickly returned to the constant-temperature room, where all observations were made. The equipment and procedures were similar to those previously used (Giese, 1938*a* and 1938*b*). Observations were made at intervals of 15–45 minutes to determine when most of the eggs had undergone a cleavage.

One drop of a sperm suspension containing about 1 part of the spermatic fluid to 200 parts of sea water was used for each 250–300 eggs contained in about 1 cc. of sea water. Even when all the eggs showed fertilization membranes after insemination, a few failed to cleave and some underwent abnormal cleavage, but the others showed normal and rhythmic cleavage and gave rise to apparently normal trochophores. Tyler (1931) has described the early development of *Urechis;* the reader is referred to his paper for details.

EXPERIMENTAL

In the first series of experiments the eggs and sperm were irradiated with a graded series of exposures 41 mm. from the center of a mercury-argon discharge tube, which emits radiations about 88 per cent of which (excepting the heat rays) are of λ 2537 A (Leighton and Leighton, 1935), to explore more rapidly the various types of effects resulting from the irradiation with ultra-violet light. The more interesting effects were then investigated, using light of two wave-lengths obtained from a monochromator, viz.,

λ 2654 A and λ 2804 A. These two lines were chosen because the first lies near the region of maximal absorption by nucleoproteins (Heyroth and Loofbourow, 1933) and the second near a maximum for cytoplasmic proteins (Coulter, Stone, and Kabat, 1935).

TABLE 1

EFFECTS OF RADIATIONS OF THE MERCURY-ARGON DISCHARGE TUBE UPON EGGS AND SPERM OF *Urechis*

Dosage in Seconds	Sperm Irradiated, Then Used To Inseminate Normal Eggs	Eggs Irradiated Not Fertilized	Eggs Irradiated, Then Fertilized with Normal Sperm
0	Normal fertilization Normal cleavage	No change observable	
4	Normal fertilization membranes Early cleavage almost normal, later somewhat abnormal	No change observable	Normal fertilization membranes Retarded and somewhat irregular cleavage Abnormal gastrulae
8	Normal fertilization membranes Early cleavage almost normal, later abnormal	No change observable	Normal fertilization membranes Retarded and irregular cleavage Small percentage motile larvae
16	Normal fertilization membranes Some inactivated Abnormal cleavage	Activation of some Membranes appear normal Cleavage of many of those activated Abnormal motile embryos	Normal fertilization membranes Abnormal cleavage Occasional motile larvae
32	Most inactivated (all in two trials) Abnormal and occasional cleavage (no cleavage in two trials)	Activation of most Cleavage of small proportion, abnormal	One-sided crinkled fertilization membranes Nuclei divide but only occasional abnormal cleavage
64	Inactivated No fertilization membranes induced	Activation; abnormal membranes on almost all Some became multinucleate Cleavage rare and abortive	One-sided crinkled fertilization membranes Nuclei divide but cleavage rare
128	Inactivated No fertilization membranes induced	Activation; abnormal membranes on most Germinal vesicle may break down but no cleavage occurs Early cytolysis	Abnormal fertilization membranes Neither nuclear nor cytoplasmic division Early cytolysis

1. *Irregular cleavage after irradiation and its causes.*—The more interesting effects of the mercury-argon lamp upon the eggs and sperm are summarized in Table 1. As can be seen from this, irradiation produces both (1) retardation of the rate of cleavage and (2) irregularities in cleavage. The irregularities in cleavage were observed to be of three types: (*a*) a greater distribution of cleavage stages (e.g., while controls seldom showed more than two stages at the same time [cf. 4- and 8-celled stages], those ir-

radiated showed three or even four stages [cf. 1-, 2-, 4-, and 8-celled stages] at the same time); (*b*) stages not normally present except transiently or occasionally, such as the 3-celled, 5-celled, 6-celled, etc., in which cleavage of certain blastomeres lags behind that of others; (*c*) completely abnormal stages in which large- and small-sized, as well as irregularly shaped, blastomeres occur. Some irregular cleavages are illustrated in Plate I, Figures 5–16.[1]

These irregularities in cleavage may be due to (1) polyspermy in some cases; (2) suppression of polar-body formation after larger dosages in others; and (3), probably in most cases, an effect of the radiation on the processes which regulate distribution of nuclear and cytoplasmic materials in cleavage as well. Thus, a large number of the eggs irradiated for 4 seconds gave rise to 3, 4, 5, or even more cells in the first cleavage. These eggs both showed polar bodies and underwent the first cleavage at about the same time as the controls. Since ultra-violet light does not generally seem to stimulate nuclear divisions, the division of the egg into more than 2 cells at about the time controls gave rise to 2 cells most probably indicates polyspermy. At higher dosages and in a small proportion of cases at lower dosages polar bodies were not observed to form (occasionally after a dose of 8 seconds, rarely after a dose of 16 seconds). In such cases irregularities in cleavage might be due to suppression of polar-body formation or such suppression in addition to the polyspermic condition. Chase (1937) has demonstrated that, when the dosage is great enough, the polar bodies are prevented from forming in *Urechis* eggs.

But, even when neither of these could occur, as when eggs were irradiated just after the first cleavage, irregularity in cleavage was observed to occur. While the effects appeared somewhat later in such cases, nonetheless, when the dosage was sufficient, the same types of irregularities occurred. In this case the irregularities must be due to interference with the processes which regulate the regular distribution of nuclear and cytoplasmic materials.

2. *Effect of ultra-violet light of* λ *2654 A and* λ *2804 A on Urechis eggs.*—If one were to use the 2-celled stage for irradiation experiments, it would be possible to avoid polyspermy and suppression of polar bodies; but, because of a fairly long latent period, it is impossible to get a measure of the true retardation of the earlier cleavages. Therefore, in the experiments with monochromatic light, eggs were irradiated 30 minutes after insemination. In this case it is possible to avoid polyspermy, but after large dosages polar-body formation is prevented.

Eggs were placed in an all-quartz cell 5.845 mm. in diameter and irradiated with light from the monochromator. The maximal intensity delivered by the arc was utilized at all times; but, since this intensity decreased as the experiments were performed, the intensity varied. For λ 2654 A the intensity varied from 2.83 to 4.25 ergs/sec/mm²; for λ 2804 A, from 3.36 to 4.95 ergs/sec/mm². The range is relatively small in each case, and the order of magnitude for the two wave-lengths comparable.

Eggs given dosages of about 70 ergs/mm² at λ 2804 A and about 65–270 ergs/mm² at λ 2654 A seemed to cleave comparably to controls; but when the dosage was increased, eggs showed retardation, and with sufficient dosage, irregularity in cleavage. Dosages up to 1,000 ergs/mm² were mainly retarding (irregularities rare at smaller dosages but appearing more frequently with increase in dosage); between 1,000 and 2,000 ergs/mm², produce irregular cleavage in many; between 2,000 and 3,000 ergs/mm², produce ir-

[1] The author is indebted to Mr. E. A. Reed for making all the photographs used in this paper.

regularities in most. Following dosages between 3,000 and 4,000 ergs/mm², only a small percentage of the eggs ever underwent cleavage, and then only abnormally, while dosages of over 4,000 ergs/mm² were followed shortly afterward by cytolysis.

The irregularities in cleavage render collecting comparative data following larger dosages very difficult. The data here presented for the larger dosages are therefore not as accurate as the data published for the sea-urchin egg.

In practically all cases data for the two wave-lengths were obtained by using eggs from the same female and sperm from the same male, since it is possible to use one animal for six experiments (draining one egg or sperm segmental organ at a time; see Fisher and MacGinitie, 1928, for the structure of *Urechis*). Seven such series of experiments were performed, using the eggs and sperm of five different males and females.

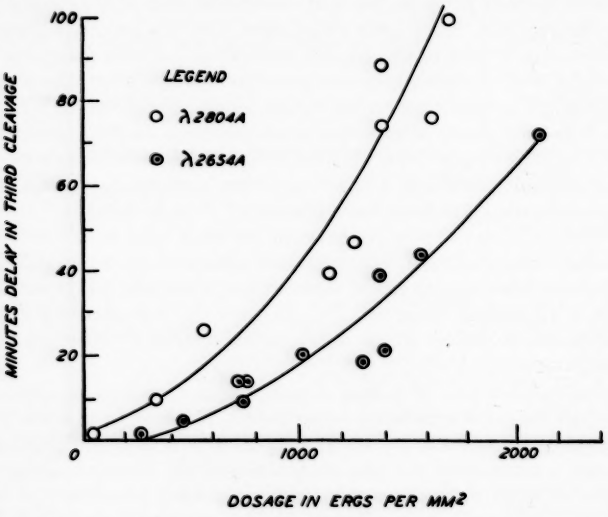

FIG. 1.—Retardation of third cleavage by irradiation with λ 2804 A in *A* and λ 2654 A in *B*

For comparison of the effect of the radiations the delay in the third cleavage was chosen, for reasons already discussed (Giese, 1938*b*). The data are plotted in text Figure 1. Study of this figure will disclose that, while there is a large spread in points, owing in part to the difficulties already noted and in part to variations in responses of eggs of different females, there is a trend indicating greater efficiency of λ 2804 A than of λ 2654 A in retarding cleavage of the eggs.

To gain an idea of the number of quanta required to produce retardation, the amount of light extinguished by the eggs was next determined by interposing a cell—first empty, then full of eggs—between the light source and the thermopile. By counting the eggs, the amount of radiation extinguished by a single egg could be determined. The details of the method are similar to those already described for the sea urchin (Giese, 1938*b*). Eggs were used in each set of extinction measurements approximately 30–50 minutes after insemination. The area of an egg extinguishing light is equivalent to the optical

sectional area. To determine this optical section, the two diameters (since some of the eggs are somewhat ellipsoidal) of 30 eggs were measured by projecting photographs of eggs taken 38 and 64 minutes after insemination; the average diameter was found to be 105.7 μ in both cases. An egg of this diameter has an optical section area of 8,758.3 μ^2. Knowing the area of a single egg and the numbers of eggs used, one can readily determine the number of layers of eggs exposed in each of the trials. The data are given in Table 2.

TABLE 2

EXTINCTION OF ULTRA-VIOLET LIGHT BY *Urechis* EGGS

Expt. No.	No. of Eggs	Layers of Eggs (L)	$1 - I^*/I_0$ for—		Comparative Extinction† at—	
			λ 2654 A	λ 2804 A	λ 2654 A	λ 2804 A
1	3,854	1.258	0.885	0.912	97.0	100
2	2,360	0.770	0.685	0.725	94.5	100
3	3,558	1.166	0.830	0.864	96.0	100

* I = intensity of transmitted light; I_0, of the incident light.

† Setting extinction at λ 2804 A = 100 in each case.

As can be seen from the table, the difference in extinction at the two wave-lengths is not very large, but it is observed in each of the experiments tried. To get an idea of the fraction of the light falling upon a single egg which is extinguished, the data for experiment No. 2 were used, and it was found that approximately 88 per cent of the incident light at λ 2654 A and 94 per cent of the light at λ 2804 A would be extinguished.

To obtain the quantity of energy extinguished by a given egg before a given effect is produced, it is merely necessary to multiply the incident energy in ergs per square millimeter required to produce a given effect by the area of the egg (8,758 μ^2 or 0.008758 mm.^{2}) and by the fraction of the incident light extinguished by the egg (approximately 94 per cent at λ 2804 A). To produce a delay in the third cleavage of about 1 hour, approximately 1,250 ergs/mm^2 are needed. This means that 1.46×10^{12} quanta have been extinguished. Of the quantity extinguished, only part are truly absorbed; part are reflected and scattered. But the order of magnitude of the quantity needed is not altered even if we should assume that half or even more than half were merely scattered.

3. *Effect of ultra-violet radiations on Urechis sperm.*—Hinrichs (1926) has described abnormal development of sea-urchin eggs fertilized by irradiated sperm. The sperm is almost a naked nucleus, and it seemed interesting to compare its resistance to ultra-violet light with that of the egg. The sperm suspension removed from a male *Urechis* was diluted to 1 : 200 with sea water in the first two series of experiments and to 1 : 1,000 in the last series.

The intensity of the light was comparable to the intensity used for the eggs. A dosage series from 158 to 3,020 ergs/mm^2 was used at λ 2654 A, and a dosage series from 189 to 4,328 ergs/mm^2 was used at λ 2804 A. Even after the largest dosages given, some sperm retained their motility and some were able to evoke membrane formation on the eggs. Longer dosages were not tried, since after about an hour sperm begin to lose potency even without irradiation at the dilutions used.

After dosages as high as 3,020 ergs/mm² for λ 2654 A and 4,328 ergs/mm² at λ 2804 A had been given the sperm, polar-body formation of eggs inseminated with the sperm and the first and second cleavages occurred in almost the normal manner and at almost the same time as in the controls. For the smallest dosages the cleavages were indistinguishable from those of the controls. But generally by the third cleavage the injurious effects of the irradiation of the sperm were quite apparent. Eggs fertilized by sperm which had been given large doses of the radiations showed at this time either (1) extremely abnormal division of at least one-half of the cells or (2) the disintegration of one-half of the cells into small globules. The effects were most pronounced and appeared earlier, the greater the dosages given. Irregular cleavage following insemination by irradiated sperm is illustrated in Figures 17–20 (Pl. I).

A few measurements of the extinction of ultra-violet light of these two wave-lengths by sperm were made and are summarized in Table 3. As can be seen from the table,

TABLE 3

EXTINCTION OF ULTRA-VIOLET LIGHT BY *Urechis* SPERM

Expt. No.	Approximate Dilution of Sperm	$1 - I/I_0$ for—		Comparative Extinction* at—	
		λ 2654 A	λ 2804 A	λ 2654 A	λ 2804 A
1	1:80	0.529	0.460	100	86.9
2	1:40	0.754	0.657	100	87.1
3	1:40	0.800	0.670	100	83.7
4	1:40	0.827	0.710	100	85.8
5	1:40	0.848	0.740	100	87.3

* Setting extinction at λ 2654 A = 100 in each case.

more light is extinguished by the sperm at λ 2654 A than at λ 2804 A, as might be expected of cells containing a large proportion of nuclear material. From this one might expect a greater action of λ 2654 A than of λ 2804 A upon the sperm. Unfortunately, from the nature of the data it is impossible to be objective, and the attempt to decide between the comparative efficiency of the two wave-lengths was abandoned. In any case, the efficiency of the two wave-lengths in producing the irregular development is of the same order of magnitude.

Ultimately the eggs fertilized with sperm given smaller dosages of radiations formed blastulae, some of which gastrulated and finally formed trochophores. Eggs fertilized with sperm given larger dosages developed abnormal swimming forms, often consisting of a large cell and, attached to one end of it, a bunch of small cells, looking, in many instances, like a balloon and a basket. Such abnormal embryos merely revolved in circles. The data are summarized and compared to the data for the eggs in Table 1.

DISCUSSION

Irradiation of *Urechis* eggs with ultra-violet light has two main effects upon cleavage of the eggs: (1) retardation of the rate of cleavage and (2) production of irregular cleavage. The retardation of cleavage is probably due to interference with reactions which

govern the rate of cleavage, while the irregularity is probably due to interference with processes which govern the regular distribution of materials. Under certain conditions irregularities may be due to polyspermy or suppressed polar-body formation as well. In the *Urechis* egg the threshold for (1) is high; thus, whereas a dosage of 1,000 ergs/mm² at λ 2804 A will produce delay at the third cleavage of about 40 minutes in *Urechis*, such a dosage produces a delay of about 2½ hours in the sea-urchin egg. The threshold for (2) in the *Urechis* egg, however, is lower than that of the sea-urchin egg, for irregularities are readily produced even at intermediate dosages, whereas sea-urchin eggs must be given more prolonged dosages to evoke comparable irregularities.

The membrane of the *Urechis* egg is quite readily altered by irradiation. Thus, when the egg is irradiated with small dosages of radiations from the mercury-argon discharge tube, the fertilization membranes form even faster than in controls; but when the dosage is larger (over 16 seconds), the membrane becomes more crinkled than in controls and tends to be one-sided. Dented eggs (see Tyler, 1932, for a discussion of the types of eggs of *Urechis*) round out quickly after insemination; those irradiated for less than 16 seconds with the discharge tube develop dents again; those irradiated for more than this dosage do not, indicating various osmotic effects. Unfertilized eggs of *Urechis* irradiated with the mercury-argon discharge tube with appropriate dosages were found to become activated (irradiation of the medium for even twenty times as long had no effect). Some of the data are given in Table 1. This activation is in striking contrast to the situation obtaining for the sea-urchin egg, where comparable dosages produced no activation (Giesé, 1938*a*).

The greater efficiency of λ 2804 A over λ 2654 A in retarding cleavage of the eggs suggests a greater effect on the cell cytoplasm than upon the nucleus, although the nucleus is undoubtedly affected as well, since nucleoproteins absorb strongly at both of these wave-lengths (Caspersson, 1936). The fact that the sperm is so highly susceptible to the radiations indicates nuclear sensitivity to both wave-lengths and suggests that the nucleus of the egg, which seems to be less affected, is probably protected at both wave-lengths by the mantle of cytoplasm which incloses it. Unfortunately, the effects on egg and sperm are qualitatively different and do not allow of direct quantitative comparison.

Following irradiation of eggs occurs a latent period before the effects are fully expressed and definitely observable, although slight retardation is early detectable. Thus, the toxic photoproducts produced by the radiations do not seem to produce a full effect until a certain time-interval has elapsed. Possibly this period is the time required for the diffusion of the toxic materials from the seat of formation. When irradiated sperm are used to inseminate normal eggs, the first two cleavages show slight retardation, the effect being expressed fully by about the third cleavage. This suggests that the units introduced by the sperm act for a time in almost a normal manner before disintegration and full expression of the effect occurs. The sperm of *Urechis* are not readily inactivated by the radiations used, but irradiated sperm produce irregularities in cleavage (generally after the second cleavage) following fairly small dosages. The sperm of the sea urchin, on the other hand, are more readily inactivated and rendered impotent, and the effect on cleavage is also more quickly expressed, than in the former case (Giese, 1939).

The number of quanta required to produce a given retarding effect in both *Urechis* and sea-urchin eggs is of the same order of magnitude, although, as already pointed

out, there are differences in susceptibility of the two eggs. The number of molecules involved is, however, in both cases very large.

The differences in response to ultra-violet radiations of the eggs and sperm of the sea urchin and of *Urechis* probably depend upon differences in their structure and physiological nature. Thus, the *Urechis* egg is larger than the sea-urchin egg (105.7 μ compared to 77.2 μ in diameter for the sea urchin). The *Urechis* egg extinguishes more of the incident light than does the sea-urchin egg (e.g., 76.3 per cent at λ 2804 A for the sea-urchin egg, 94 per cent for *Urechis*). The egg of the sea urchin contains a yellowish pigment; the egg of *Urechis*, a granular reddish pigment. The egg of *Urechis* has a more rapid rate of cleavage and of differentiation than that of the sea urchin. The more active egg may thus repair injuries more quickly than the less active one. On the other hand, the cleavage of the *Urechis* egg is rendered irregular with smaller dosages of radiations than is the sea-urchin egg. This may indicate differential susceptibility in the two eggs of the reaction chains governing the rate of cleavage, on the one hand, and the regularity of cleavage, on the other.

The effects above enumerated, as well as other effects previously discussed, indicate that the action of the radiations on the eggs is a summation of the effect on various cell structures and functions. The vast number of quanta involved in producing the effects also suggests a complex series of reactions following the absorption of the light by the cell.

SUMMARY

1. Following irradiation with ultra-violet light, the eggs of *Urechis* were found to undergo retarded and irregular cleavage.

2. Retardation, as well as irregularity, increased with increase in dosage.

3. Irregularities were of three kinds: (1) presence of more developmental stages at one time, (2) lag in cleavage of some blastomeres, and (3) unequal and irregular divisions.

4. Retarded and, following larger dosages, irregular cleavage occurred whether the unfertilized egg, the egg 30 minutes after insemination, the 2-celled stage, or the sperm were irradiated with sufficient dosages.

5. A latent period followed the irradiation before the effects of the absorbed radiation were fully expressed.

6. Activation occurred when unfertilized eggs were given certain dosages of radiations from the mercury-argon discharge tube.

7. Polyspermy occurred when unfertilized eggs were given certain dosages and were subsequently inseminated.

8. Of the two wave-lengths, λ 2804 A and λ 2654 A, the first was somewhat more effective in causing retardation than the second.

9. The extinction of radiations by the *Urechis* eggs is of the same order of magnitude for each of these wave-lengths, being slightly larger for λ 2804 A than for λ 2654 A.

10. The extinction of radiations by sperm is greater at λ 2654 A than at λ 2804 A.

11. The number of quanta needed to produce retardation of cleavage of the eggs is large, being of the order of magnitude of 10^{12} to produce a delay of about 1 hour in the third cleavage after irradiation with λ 2804 A.

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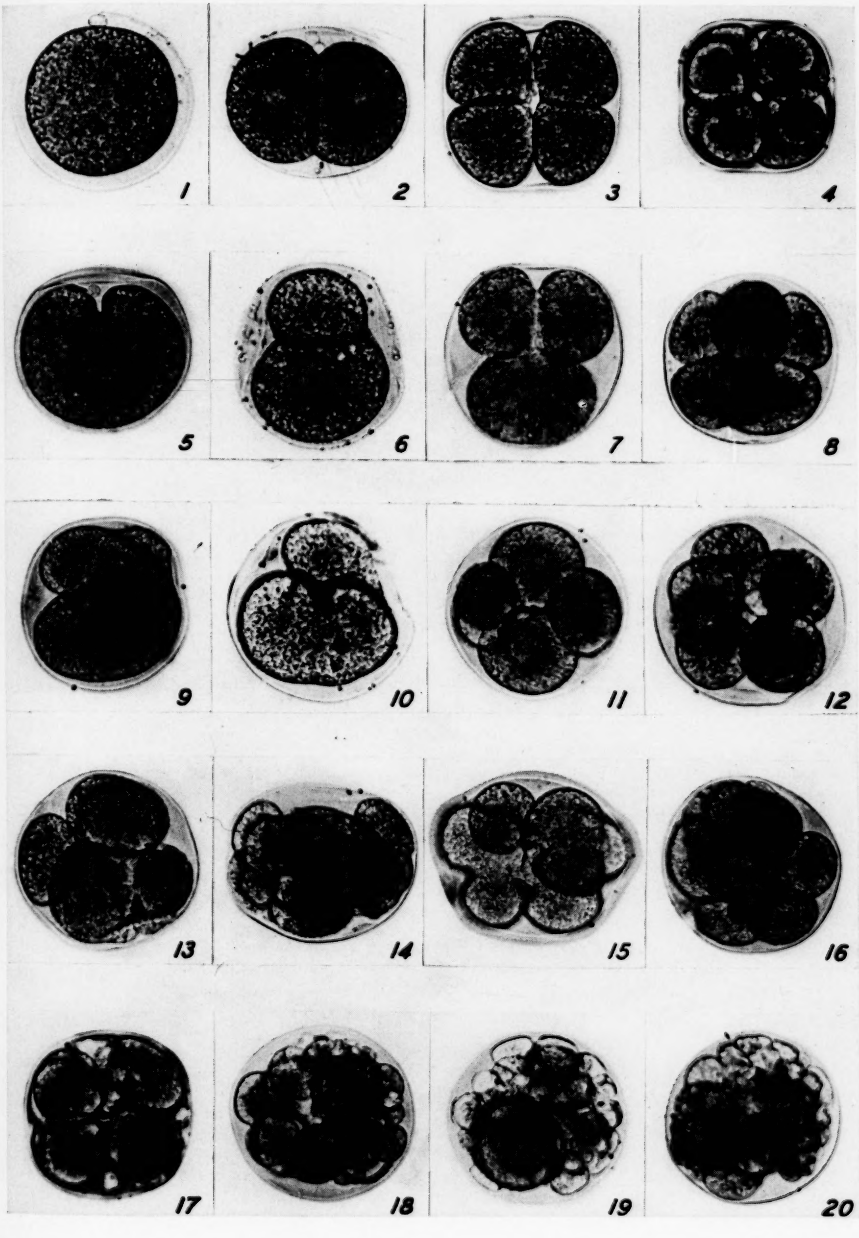
PLATE I

Figs. 1–4.—Stages in normal development.
Figs. 5–16.—Abnormalities in development of irradiated eggs.
Figs. 17–20.—Abnormalities in development of eggs fertilized by irradiated sperm.

PLATE I



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